Rejections Under 35 U.S.C. §§102(b) and 103

In the Office Action mailed November 14, 1996, the Examiner rejected Claims 7, 8, and 16 under 35 U.S.C. §102(b) as anticipated by or under 35 U.S.C. §103 as obvious over the article by Lee, Francis K., *et al.*, "Detection of Herpes Simplex Virus Type 2-Specific Antibody with Glycoprotein G," *J. Clin. Microbiol.* 22(4):641-644 (October 1985) or the article by Lee, Francis K., *et al.*, "A Novel Glycoprotein for Detection of Herpes Simplex Virus type 1-specific Antibodies," *J. Virol. Methods* 14:111-118 (1986). The Examiner noted that the cited articles teach the production and use of herpes simplex virus gG-1 and gG-2 antigens, the rejected claims are product-by-process claims, and patentable distinctions between the material, structural, and functional characteristics of the claimed compositions and the prior art compositions had not yet been demonstrated. Applicants respectfully submit that the amendments to the claims overcome the rejection.

Claims 7 and 8 have been amended to clarify that the claimed herpes simplex virus gG-1 and gG-2 antigens are **recombinant** antigens. Claim 16 also specifies that the claimed antigens are recombinant.

Lee *et al.*, *J. Clin. Microbiol.* 22:641-644 (1985), teach the use of purified herpes simplex virus type 2-specific glycoprotein (gG-2) in an immunodot enzymatic assay for the detection of HSV-2 antibodies in human serum. The gG-2 antigen used by Lee *et al.* (1985) was purified from HSV-2-infected HEp-2 cells using immunoaffinity chromatography columns containing the anti-gG-2 mouse monoclonal antibodies H966 and H1206. Applicants respectfully submit that Lee *et al.* (1985) fail to disclose a **recombinant** gG-2 antigen.

Lee *et al.*, *J. Virol. Methods* 14:111-118 (1986), teach the purification of herpes simplex virus type 1-specific glycoprotein (gG-1) using the mouse monoclonal antibody H1379-2. Applicants respectfully submit that Lee *et al.* (1986) fail to disclose a **recombinant** gG-1 antigen.

Applicants respectfully submit that **recombinant** gG-1 and gG-2 glycoprotein antigens are structurally different from gG-1 and gG-2 glycoprotein antigens produced by the herpes simplex virus types 1 and 2. As explained in the present specification, although the recombinant and gG-1 and gG-2 glycoproteins produced by the method described in the present application react with the same antibodies that react with non-recombinant HSV gG-1 and gG-2 glycoproteins, the recombinant proteins are different in that they are differently glycosylated than the non-recombinantly produced glycoproteins. (See page 25, line 20 to page 26, line 7 of the

present specification.) Therefore, by describing the gG-1 and gG-2 antigens as "recombinant" antigens, they are by definition structurally different from the naturally-produced glycoproteins described by Lee *et al.* (1985) and Lee *et al.* (1986). Applicants note that the Examiner has provided no reference teaching the production of **recombinant** HSV glycoproteins gG-1 and gG-2 using **any** vector.

Structural differences between the claimed recombinant HSV glycoproteins gG-1 and gG-2 and naturally produced gG-1 and gG-2 are shown in Figures 3 and 4 of the scientific article by Sanchez-Martinez and Pellett, "Expression of HSV-1 and HSV-2 Glycoprotein G in Insect Cells by Using a Novel Baculovirus Expression Vector", *Virol.* 182:229-238 (1991). This article describes experimental data generated by the applicants that was included in Figures 3 and 4 and on page 21, lines 3-16 and page 23, lines 19-30 of the present specification. A new copy of this article is enclosed as Exhibit 1 so that the Examiner can more clearly see the electrophoretic bands. For example, Figure 3C of the Sanchez-Martinez and Pellett article provides an immunoblot analysis comparing the molecular weights of proteins extracted from Sf9 cells infected with the baculovirus vector AcDSMgG-1 containing the gG-1 gene (recombinant gG-1) with proteins extracted from HEp-2 cells infected with HSV-1 (non-recombinant gG-1). The recombinant gG-1 shows strong bands at 42 and 43 kDa, whereas the non-recombinant gG-1 shows a smear between 50 and 57 kDa. Similarly, Figure 4D of the Sanchez-Martinez and Pellett article provides an immunoblot analysis comparing the molecular weights of proteins extracted from Sf9 cells infected with the baculovirus vector AcDSMgG-2 containing the gG-2 gene (recombinant gG-2) with proteins extracted from HEp-2 cells infected with HSV-2 (non-recombinant gG-2). The recombinant gG-2 shows distinct bands at 107, 118, 128 and 143 kDa, whereas the non-recombinant gG-2 shows a smear between 78 and 118 kDa. It is well understood by those skilled in the art that proteins having different immunoblot band patterns are considered structurally different.

Applicants respectfully submit that because the claimed recombinant HSV glycoproteins gG-1 and gG-2 are different from non-recombinant gG-1 and gG-2 antigens, the claimed antigens are not anticipated by the cited references and the rejection under 35 U.S.C. §102(b) should be withdrawn.

The Examiner rejected Claims 7, 8, and 16 under 35 U.S.C. §103 as obvious over the articles of Lee *et al.*, (1985) or Lee *et al.* (1986) alone and in view of the article of Luckow,

Verne A., *et al.*, "Trends in the Development of Baculovirus Expression Vectors," *Bio/Technology*, 6:47-55 (January 1988) or the article of Matsuura, Yoshiharu, *et al.*, "Baculovirus Expression Vectors: the Requirements for High Level Expression of Proteins, Including Glycoproteins," *J. Gen. Virol.*, 68:1233-1250 (1987). The Examiner stated that the monoclonal antibodies used by Lee *et al.* (1985) and (1986) appeared to be the same as those described on page 7 of the present specification and that one skilled in the art would have been motivated to express the HSV gG-1 and gG-2 glycoproteins in a baculovirus system as taught by Luckow *et al.* and Matsuura *et al.* for other proteins.

Lee *et al.* (1985) and (1986) teach the use of affinity chromatography-purified HSV gG-1 and gG-2 glycoproteins as described above.

Matsuura *et al.* teach that the polyhedrin gene promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) is useful for high level expression of a glycoprotein of lymphocytic choriomeningitis virus. Matsuura *et al.* suggest that the synthesis is related to the integrity of the 5' non-coding region of the polyhedrin gene. Matsuura *et al.* teach that the baculovirus vector pAcYM1 provides expression of the glycoprotein precursor (GPC) of lymphocytic choriomeningitis virus amounting to approximately 20% of the total cellular protein.

Luckow *et al.* is a review article directed to baculovirus expression vectors. Luckow *et al.* describe numerous factors affecting the expression of foreign genes by baculovirus vectors including optimizing placement of the foreign gene within the transfer vector. Applicants respectfully submit that Luckow *et al.* fail to teach use of the vector pAcDSM, fail to teach insertion of the foreign gene precisely at the translation initiation codon of the polyhedrin gene, without either missing any nucleotide present in said initiation codon or introducing any extraneous nucleotide at the initiation codon site, and fail to teach or suggest expression of herpes simplex virus antigens.

As discussed above, the claims of the present application are directed to **recombinant** HSV gG-1 and gG-2 antigens produced from a novel baculovirus vector. Not only are the recombinant antigens structurally different from **naturally** produced gG-1 and gG-2 antigens as described above, but the claimed recombinant antigens are produced in greater quantities thereby yielding a purer product than recombinant gG-1 and gG-2 antigens produced using vectors such as vaccinia vectors or alternative baculovirus systems such as the pAc373 baculovirus system. In addition, the claimed recombinant gG-1 and gG-2 proteins are produced in

the absence of the other 80 herpes simplex virus proteins that are produced naturally by herpes simplex virus type 1 or herpes simplex virus type 2-infected mammalian cells. Nearly every gene encoded by herpes simplex virus type 1 has a genetic counterpart in herpes simplex virus type 2, and the protein products of these homologous genes have significant antigenic cross-reactivity, except for gG-1 and gG-2. This is the basis of the unique utility of these proteins for construction of HSV type-specific serologic assays. The claimed proteins are therefore much purer and less likely to be contaminated by HSV-type cross-reactive antigens than gG-1 and gG-2 proteins produced by other means.

The Examiner stated that Lee *et al.* (1985) and Lee *et al.* (1986) use the monoclonal antibodies H1206 and H1379 in a detection assay to measure HSV-2 and HSV-1. The Examiner then concluded that applicants use the same antibodies, described on page 7 of the present specification, to purify the claimed **recombinant** proteins. Applicants agree that they used the same antibodies as Lee *et al.* (1985) and Lee *et al.* (1986), but applicants respectfully submit that they used the antibodies for a different purpose. As described above, Lee *et al.* (1985) and Lee *et al.* (1986) use the antibodies to prepare affinity chromatography columns, which are used to isolate gG-1 or gG-2 antigens as follows. Extracts from cells infected with HSV-1 or HSV-2 are passed through columns packed with a resin to which is bound the type-specific antibodies. The gG-1 antigen or gG-2 antigen present in the extract couples to the type-specific antibodies on the column as the extract passes through the column. The column is washed to remove any residual cellular components, and the bound antigen is then eluted from the column and collected. In contrast, the present specification states, on page 7, lines 27-30, that monoclonal antibodies, designated H1379 and H1206, were obtained from Dr. Lenore Pereira. Use of the monoclonal antibodies in an immunoblot assay to analyze gG-1 or gG-2 protein production from the baculovirus system is described in the specification on page 12, lines 22-26. Use of the monoclonal antibody H1206 for detection purposes is also taught on page 21, line 22 to page 22, line 8 and the results shown in Figures 4A and 4B. Applicants respectfully submit that the present specification describes the use of monoclonal antibodies to confirm that the proteins expressed from the baculovirus systems were recombinant gG-1 and gG-2. Applicants are not using the monoclonal antibodies in an affinity column for purposes of antigen purification. The mere fact that the recombinant and non-recombinant antigens bind to the same monoclonal antibody does not demonstrate that the proteins

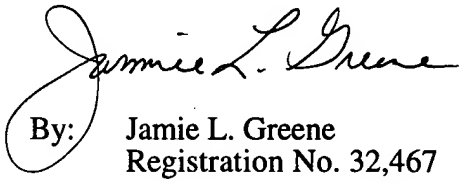
are identical, it indicates only that the recombinant and non-recombinant proteins share an epitope with which the monoclonal antibodies cross-react.

Conclusion

In conclusion, applicants respectfully submit that Claims 7, 8 and 16 are novel and non-obvious in view of the references cited by the Examiner.

Applicant maintains that the claims are in condition for allowance. A Notice of Allowance is therefore respectfully solicited. If the Examiner believes any informalities remain in the application that may be corrected by Examiner's Amendment, or there are any other issues that can be resolved by telephone interview, a telephone call to the undersigned attorney at (404) 818-3773 is courteously solicited.

Respectfully submitted,
JONES & ASKEW



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Expression of HSV-1 and HSV-2 Glycoprotein G in Insect Cells by Using a Novel Baculovirus Expression Vector

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Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) glycoprotein G (gG-1 and gG-2) were expressed in insect cells from recombinant baculoviruses (AcDSMgG-1 and AcDSMgG-2, respectively) constructed using a novel baculovirus transfer vector, pAcDSM. This vector allows the coding region of a foreign gene to be precisely linked to the baculovirus polyhedrin gene at the translation initiation site and retains the native polyhedrin translation initiation environment. Fourfold more gG-1, with a higher ratio of glycosylated to unglycosylated product, was produced by AcDSMgG-1 than by Ac373'gG-1, a recombinant baculovirus which differs from AcDSMgG-1 by the presence of 21 extraneous nucleotides in the 5' nontranslated sequence. gG-1 and gG-2 expressed in recombinant baculovirus-infected insect cells undergo cotranslational N-linked glycosylation, but the overall processing of the proteins differs from that observed in HSV-1- or HSV-2-infected cells. Despite these differences, baculovirus-expressed gG-1 and gG-2 were recognized in a HSV type-specific manner by human serum specimens. © 1991 Academic Press, Inc.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are genetically very similar (Kieff *et al.*, 1972; Ludwig *et al.*, 1972), resulting in extensive antigenic cross-reactivity (reviewed by Nahmias and Dowdle, 1968; and Honess and Watson, 1977). This cross-reactivity has resulted in many serologic tests for distinguishing prior infection with HSV-1 from that with HSV-2 being laborious as well as frequently inconclusive, particularly with specimens containing antibodies to both viruses (Nahmias *et al.*, 1970).

The identification of glycoprotein G as a type-specific antigen (gG-1) (Ackermann *et al.*, 1986; Richman *et al.*, 1986) (and gG-2) (Roizman *et al.*, 1984; Marsden *et al.*, 1984) allowed the development of accurate type-specific serologic assays (Lee *et al.*, 1985, 1986; Nahmias *et al.*, 1986; Ashley *et al.*, 1988). Studies using these assays have clearly demonstrated the accuracy that can be obtained using reactivity with gG-1 and gG-2 as the basis for the assay, but because of difficulties in preparing and standardizing reagents, the tests have been performed routinely in few laboratories. To obtain abundant supplies of well-characterized antigen for use in serologic tests and in studies of the host immune response to gG, and to bypass large-scale culturing of pathogenic agents, we have expressed gG-1 and gG-2 in the baculovirus expression system (reviewed by Luckow and Summers, 1988).

Although high levels of gene expression have been obtained using the baculovirus system, in only a few cases has the level of gene expression approached that of native polyhedrin. Kozak (1981) has shown that sequences immediately surrounding the translation initiation site can have a profound effect on the efficiency of translation initiation. We hypothesized that at least some of the differences in the level of gene expression between native polyhedrin and foreign genes inserted in its place may be due to missing or extraneous nucleotides in the vicinity of the translation initiation codon in vectors currently in use (Smith *et al.*, 1985; Matsuura *et al.*, 1987; Luckow and Summers, 1989). Thus the most efficient expression of a foreign gene in this system might occur if the native polyhedrin sequences controlling regulation of transcription and translation embedded in the 5' nontranslated leader sequence were unaltered and joined to the coding region of the foreign gene precisely at the translation initiation site, with no missing or extraneous nucleotides.

We describe in this report (i) the creation of a baculovirus transfer vector that fulfills the above requirements, (ii) its application for the construction of recombinant baculoviruses expressing gG-1 and gG-2 in insect cells, and (iii) the characterization and type specificity for HSV antibodies in human serum specimens of baculovirus-expressed gG-1 and gG-2.

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MATERIALS AND METHODS

Cells and viruses

Wild-type and recombinant AcNPV were grown and assayed in a continuous ovarian cell line (Sf9) derived from *Spodoptera frugiperda* (fall armyworm), as previously described (Summers and Smith, 1987). Sf9 cells (ATCC No. CRL 1711) were obtained from the American Type Culture Collection (Rockville, MD). Wild-type AcNPV and the gene transfer vector pAc373 (Smith *et al.*, 1985) were obtained from Dr. Max Summers, Texas A&M University (College Station, TX). HSV-1(F) and HSV-2(G) (Ejercito *et al.*, 1968) were obtained from Dr. Bernard Roizman, University of Chicago (Chicago, IL) and grown and propagated as described (Morse *et al.*, 1977). Monoclonal antibodies specific for gG-1 (H1379) (Lee *et al.*, 1986) and specific for gG-2 (H1206) (Lee *et al.*, 1985) were obtained from Dr. Lenore Pereira, University of California, San Francisco (San Francisco, CA).

DNA manipulations

DNA manipulations were carried out essentially as described (Maniatis *et al.*, 1982). Restriction endonucleases and T4 DNA polymerase were purchased from New England BioLabs (Beverly, MA), mung bean exonuclease from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), and T4 DNA ligase from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Oligonucleotides were synthesized in a Model 380 DNA synthesizer from Applied Biosystems (Foster City, CA).

Construction of modified gene transfer vectors

pPP-2. pPP-1 was constructed by digesting pUC8 (Vieira and Messing, 1982) with *Nar*I and filling in the overhanging 5′ nucleotides using T4 DNA polymerase, followed by self-ligation, resulting in the elimination of the *Nar*I site. A synthetic oligoduplex AB (Fig. 1A) was ligated between the *Eco*RI and the *Hin*dIII sites of pPP-1, to obtain pPP-2. This vector can be used to construct gene fusions at any of the three reading frames by using blunt-end ligations at the appropriate site. The fused gene would then be removed from the vector by digestion at the flanking *Eco*RI and *Hin*dIII sites and inserted into a baculovirus transfer vector such as pAc373 at the desired site, using blunt ends if necessary.

pAcDSM. pAc373 was digested to completion with *Sal*I and *Kpn*I. The DNA fragment spanning the region between 3.18 and 4.43 kilobase pairs (kbp) in the coordinate system of Summers and Smith (1987) was purified from agarose and inserted into pUC19 (Norrander *et al.*, 1983) that had previously been digested with the same enzymes. The resulting plasmid (pDM1) was linearized by digestion with *Ava*I. After PEG precipitation (Sadhu and Gedamu, 1988), 5′ overhanging nucleotides were removed by digestion with 150 units of mung bean exonuclease/μg of DNA to obtain blunt ends, followed by *Kpn*I digestion. A synthetic oligoduplex, V78 (Fig. 1A), was inserted between the *Kpn*I site and the blunt end (nucleotide −9 of the 5′ leader sequence of the polyhedrin gene) of pDM1 to obtain pDM2. The fidelity of the construct was checked by nucleotide sequencing using primers flanking the cloned fragment. pDM2 was digested with *Eco*RV and *Kpn*I. The resulting 118-bp fragment was ligated to pAc373 previously digested with the same enzymes to obtain the transfer vector pAcDSM.

In order to use pAcDSM, it is digested with *Pst*I, treated with T4 DNA polymerase to trim the 3′ overhanging nucleotides to a blunt-ended C at position −9 of the polyhedrin 5′ nontranslated leader sequence, and then digested at another restriction site in the polylinker using an enzyme which generates a cohesive terminus, allowing for efficient directional insertion of a foreign gene (Fig. 1B). The foreign gene is modified for compatibility with the vector by assembling a segment of DNA containing, from 5′ to 3′, the nucleotides −8 to −1 of the 5′ nontranslated leader sequence of the polyhedrin gene, the translation initiation codon, the coding region of the foreign gene, the downstream region through the polyadenylation signal, and a unique restriction site compatible with one in the transfer vector's polylinker. Segments of DNA containing these features can be constructed by using standard cloning techniques and synthetic oligonucleotides as was done here, by total synthesis using long synthetic oligonucleotides, or by using the polymerase chain reaction (Saiki *et al.*, 1985) with appropriately tailed primers. When the modified segment of DNA is inserted into pAcDSM prepared as described above, the C in position −9 of the polyhedrin 5′ nontranslated leader sequence is linked to the first nucleotide of the modified gene (C in position −8), regenerating the 5′ leader sequence of the polyhedrin gene.

Cloning gG-1 and gG-2 into baculovirus transfer vectors

For convenience, the nucleotide sequence coordinates reported for the genomic sequence of HSV-1 strain 17 (McGeoch *et al.*, 1988) and for the *Hin*dIII L fragment of HSV-2 strain HG52 (McGeoch *et al.*, 1987) are used throughout this paper. HSV-1 strain F (HSV-1(F)) and HSV-2 strain G (HSV-2(G)) DNAs were separately digested to completion with *Bam*HI and shotgun cloned into pUC19. Plasmids carrying HSV-1(F) *Bam*HI

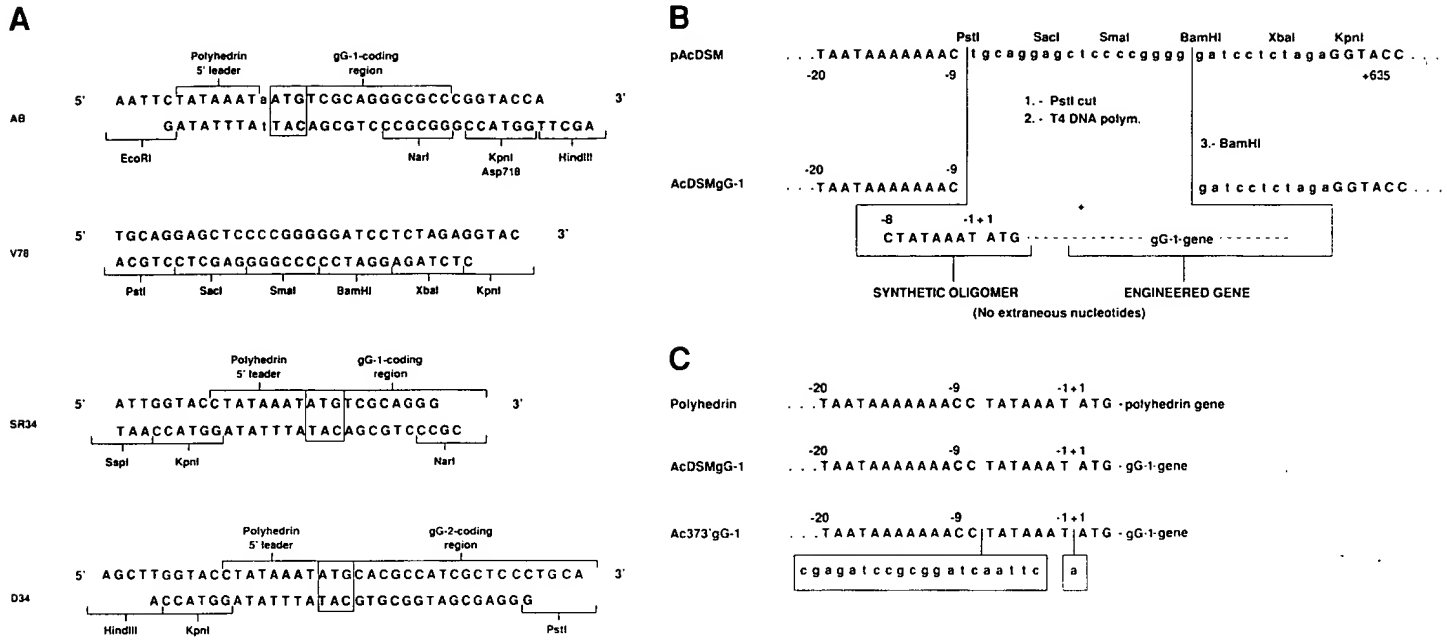

FIG. 1. (A) Synthetic oligomers used in the construction of the baculovirus gene transfer vectors and the gG-1- and gG-2-expressing recombinant baculoviruses. Oligoduplex AB was used in the construction of pPP-2, oligoduplex V78 for pAcDSM, oligoduplex SR34 for AcDSMgG-1, and oligoduplex D34 for AcDSMgG-2. Relevant restriction endonuclease sites are indicated. (B) Schematic representation of the method of inserting a foreign gene into the transfer vector pAcDSM. (C) Comparison of nucleotide sequences in the 5' nontranslated region of the wild-type baculovirus (AcNPV) and the recombinant viruses Ac373'gG-1 and AcDSMgG-1. Extraneous nucleotides relative to the wild-type polyhedrin sequence are boxed. The a preceding the initiation codon in Ac373'gG-1 was incorporated into the construct based on a published sequence (Hooft Van Iddekinge *et al.*, 1983) later found to be in error (Howard *et al.*, 1986).

J (pH1F-110, nucleotides 136,285 to 142,742) and HSV-2(G) *Bam*HI L (pH2G-112, nucleotides 2356 to 6894) fragments, which contain the intact gG-1 and gG-2 genes, respectively, were used as the starting point for engineering the genes for compatibility with the transfer vectors.

gG-1 insertion into pPP-2. pH1F-110 was digested with *Sph*I to remove nucleotides 137,617 to 142,742 of the HSV-1 fragment (removing two of the three *Nar*I sites in the insert) and ligated to itself. The resulting plasmid (pH1F-1001) was digested with *Nar*I and religated to itself, deleting nucleotides 136,285 to 136,749 and a small portion of the vector, resulting in pH1F-1002. This plasmid was digested with *Nar*I and *Hin*dIII, and the 873-bp fragment carrying the nearly complete gG-1 gene (nucleotides 136,749 to 137,622) was ligated to plasmid pPP-2, which had previously been digested with *Nar*I and *Hin*dIII. The resulting plasmid (pH1F-1011) was digested with *Eco*RI and *Hin*dIII. The fragment containing the modified gG-1 gene was purified from an agarose gel, made blunt-ended with T4 DNA polymerase, and ligated to pAc373, which had been digested with *Bam*HI and made blunt-ended by treatment with T4 DNA polymerase. A plasmid containing the gG-1 gene inserted in the proper orientation was designated pAc373'gG-1.

gG-1 insertion into pAcDSM. The 871-bp fragment between the *Nar*I and the *Sph*I sites of pH1F-1002 (nucleotides 136,749 to 137,620), carrying the nearly complete gG-1 gene, was ligated to pUC18 that had previously been digested with the same enzymes. The resulting plasmid, pSR1, was digested with *Nde*I and *Bam*HI, producing a 944-bp fragment that was ligated to pUC9 that had been previously digested with the same enzymes, resulting in pSR3. A synthetic oligoduplex, SR34 (Fig. 1A), was ligated between the *Ssp*I and the *Nar*I sites of pSR3. The resulting construct, plasmid pSR5, was successively incubated with *Kpn*I, T4 DNA polymerase, and *Bam*HI and ligated with the vector pAcDSM that had previously been successively reacted with *Pst*I, T4 DNA polymerase, and *Bam*HI. The resulting plasmid was designated pAcDSMgG-1.

gG-2 insertion in pAcDSM. pH2G-112 was digested with *Bam*HI and *Pvu*II to obtain a 3779-bp fragment (nucleotides 2356 to 6135) that was purified from agarose and ligated with pUC19 that had been digested with *Bam*HI and *Ssp*I. The resulting plasmid, pDS1, was digested with *Pst*I and *Hin*dIII and ligated with a synthetic oligodupex, D34 (Fig. 1A), to generate pDS2. The 1316-bp fragment (nucleotides 2515 to 3831) resulting from digesting pH2G-112 with *Hin*cII was purified from agarose and successively reacted with *Bst*NI,

T4 DNA polymerase, and *Sty*I. The resulting 161-bp fragment (nucleotides 2859 to 3020) was purified from agarose and then ligated to pDS2, which had previously been successively incubated with *Pst*I, T4 DNA polymerase, and *Sty*I. The resulting plasmid, pDS6, was digested with *Ssp*I and *Xho*I and ligated to an agarose-purified 2148-bp fragment obtained by digesting pH2G-112 with *Ssp*I and *Xho*I (nucleotides 2983 to 5131). The resulting plasmid, pDS7, was digested with *Hin*dIII and *Xho*I. The resulting 2311-bp fragment (carrying the complete gG-2 gene plus flanking plasmid-derived sequences) was ligated with pUC9 that had been digested with *Hin*dIII and *Sal*I. The resulting plasmid, pDS8, after successive reactions with *Kpn*I, T4 DNA polymerase, and *Bam*HI, was ligated with pAcDSM that had been successively reacted with *Pst*I, T4 DNA polymerase, and *Bam*HI to obtain pAcDSMgG-2.

Transfection and selection of recombinant baculoviruses

Procedures for transfection, selection of recombinant baculoviruses, and virus titration were performed as described (Summers and Smith, 1987). Recombinant viruses were plaque purified at least five times. Proper insertion of the transferred genes into the baculovirus genome was confirmed by blot hybridization analysis of the recombinant virus genomes (data not shown).

Protein analysis by immunoblots

Sf9 cells were grown either in Hink's medium supplemented with 10% fetal calf serum according to the method of Summers and Smith (1987) or in serum-free medium (Excell 400, J. R. Scientific, Woodland, CA) and infected with recombinant or wild-type baculoviruses at a multiplicity of infection of 10 PFU/cell in 25-cm^2 flasks at 27°. After 1.5 hr, the inoculum was removed and replaced with fresh medium. At the appropriate times postinfection, the cells were scraped from the flask and harvested by centrifugation for 10 min at 1500 *g* at 4°. The pellet was resuspended in disruption buffer (5.7 *M* urea, 2.8% SDS, and 1.8 *M* 2-mercaptoethanol), sonicated for 30 sec (output control 4, duty cycle 50%) in a cup horn sonicator (Model W-375, Heat Systems-Ultrasonic, Inc., Farmingdale, NY), and heated for 3 min at 95°. Proteins were separated by electrophoresis in polyacrylamide gels (Laemmli, 1970) (acrylamide to bis-acrylamide ratio of 37.5:1) and then electrically transferred onto nitrocellulose paper (BA85, Schleicher and Schuell) (Towbin *et al.*, 1979) using 0.1% SDS in the transfer buffer. Blots were incubated for 1 hr in blotto (5% skim milk, 0.01 *M* phosphate-buffered saline, pH 7.4, and 0.05% Tween 20) and then incubated for 1 hr in fresh blotto containing the appropriate dilution of either human serum or monoclonal antibody. After three 10-min washes in 0.05% Tween 20 in phosphate-buffered saline, blots were incubated with alkaline phosphatase-conjugated goat anti-human or alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Rockville Centre, NY) in 0.05% Tween 20 in phosphate-buffered saline for 2 hr, washed three times for 10 min with the same buffer, and developed with *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Bio-Rad) according to the vendor's protocol.

RESULTS

Vector construction

Two gene transfer vectors were constructed, pPP-2, which incorporates nucleotides −7 to −1 of the 5′ nontranslated leader sequence of the polyhedrin gene missing in the widely used transfer vector pAc373 (Smith *et al.*, 1985) but which results in recombinant baculoviruses containing 21 extraneous nucleotides in this region, and pAcDSM, which allows the construction of recombinant baculoviruses with the 5′ nontranslated leader sequence of the polyhedrin gene joined precisely to the coding region of the foreign gene at the translation initiation codon, with no missing or extraneous nucleotides. pPP-2 was constructed early in the course of these studies. It is awkward to use and offers few advantages relative to other currently available vectors. Its description is included here because it was used to construct the recombinant baculovirus Ac373′gG-1.

Construction of recombinant baculoviruses expressing gG-1 and gG-2

As described under Materials and Methods, recombinant baculoviruses Ac373′gG-1 and AcDSMgG-1 expressing gG-1 were constructed using pPP-2 in conjunction with pAc373 and pAcDSM, respectively. The nucleotide sequence in the vicinity of the translation initiation codon of these viruses is shown in Fig. 1C. A recombinant baculovirus expressing gG-2, AcDSMgG-2, was created using pAcDSM.

Synthesis and processing of baculovirus-expressed gG-1

Replica immunoblots of extracts from recombinant-infected, wild-type-infected, or uninfected Sf9 cells were reacted with either a human serum specimen that had been identified as HSV-1-positive and

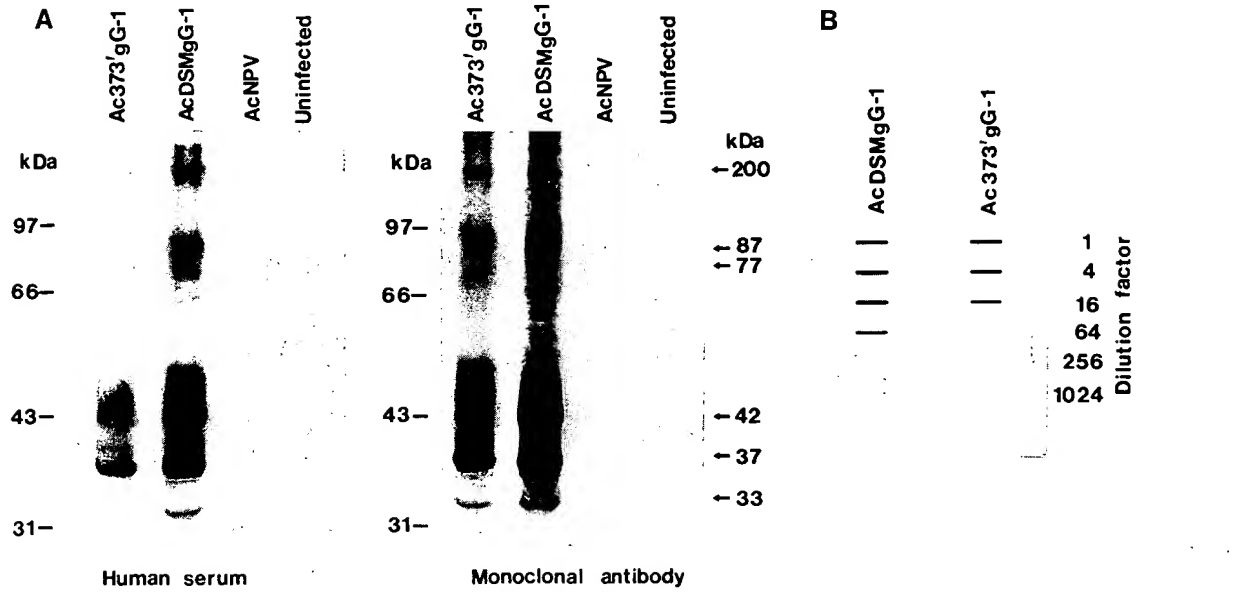

FIG. 2. Reaction of baculovirus-expressed gG-1s with antibodies. (A) Proteins extracted at 100 hr p.i. from equal numbers of Ac373'gG-1-, AcDSMgG-1-, and AcNPV-infected or uninfected Sf9 cells were separated by SDS–PAGE in 11% gels, transferred to nitrocellulose membranes, and then tested with the indicated antibodies. The positions of the molecular mass standards are shown on the side of each panel, (phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; and carbonic anhydrase, 31 kDa). The apparent molecular mass of gG-1-related species (arrows) is indicated. (B) Quantitative comparison of the amount of gG-1 expressed in Sf9 cells by the recombinants Ac373'gG-1 and AcDSMgG-1. Cell extracts similar to those used in A were fourfold serially diluted in 0.01 *M* phosphate-buffered saline, pH 7.4, bound to a nitrocellulose membrane using a slot-blot apparatus, and reacted with gG-1-specific monoclonal antibody (H1379).

HSV-2-negative using an HSV type-specific indirect hemagglutination assay (IHA) (Bernstein and Stewart, 1971) or a monoclonal antibody specific for gG-1 (H1379) (Lee *et al.*, 1986) (Fig. 2A). None of the antibodies reacted with proteins in the lanes containing proteins from AcNPV-infected or uninfected cells, except for a weak reaction between some human serum specimens and polyhedrin in AcNPV-infected cells. The pattern of reactivity with both antibodies was identical in the lanes containing the gG-1 recombinant-infected cell extracts (lanes Ac373'gG-1 and AcDSMgG-1). The major reacting bands appeared at 37 and 42 kDa apparent molecular mass (37K and 42K) within a region of diffuse reactivity between 36 and 48 kDa apparent molecular mass.

Expression of the recombinant gG-1s differed in two respects. (i) The intensity of the reaction with both antibodies was higher in extracts of cells infected with AcDSMgG-1 than with Ac373'gG-1 (Fig. 2A). (ii) In extracts of Sf9 cells infected with AcDSMgG-1, 42K reacted more than 37K. In extracts of Sf9 cells infected with Ac373'gG-1, the opposite was true, with 42K being very faint.

Slot-blot analysis was used to eliminate difficulties inherent in quantifying multiple diffuse bands in electropherograms. The intensity of the reaction was about fourfold greater in extracts harvested from cells infected with AcDSMgG-1 than with Ac373'gG-1 at either 100 hr p.i. (Fig. 2B) or 72 hr p.i. (data not shown). Thus more gG-1 was expressed from the construct that mimicked the polyhedrin 5' nontranslated leader sequence (AcDSMgG-1) than from the construct that contained 21 extraneous nucleotides in this region (Ac373'gG-1).

We monitored the levels of gG-1 production and the ratios of intensity between the two major gG-1 bands in the two recombinants as a function of time after infection (Fig. 3A). The major gG-1 bands (37K and 42K) were first detected at 36 hr p.i. At all time points, more gG-1 was detected in the lanes containing extracts from cells infected with AcDSMgG-1 (Fig. 3A, lanes b). The maximum expression with both recombinants occurred at about 72 hr p.i. As was previously seen at 100 hr p.i., at all time points 42K was more abundant than 37K in AcDSMgG-1-infected cells (lanes b), while in Ac373'gG-1-infected cells (lanes a), 37K was more abundant than 42K. This indicates that the difference in processing of gG-1 may be due to differences between the transfer vectors. Similar patterns and levels of synthesis were observed in two independently plaque-purified progeny of the transfection that produced Ac373'gG-1 (data not shown).

Treatment of AcDSMgG-1-infected Sf9 cells with tunicamycin, an inhibitor of a precursor necessary for

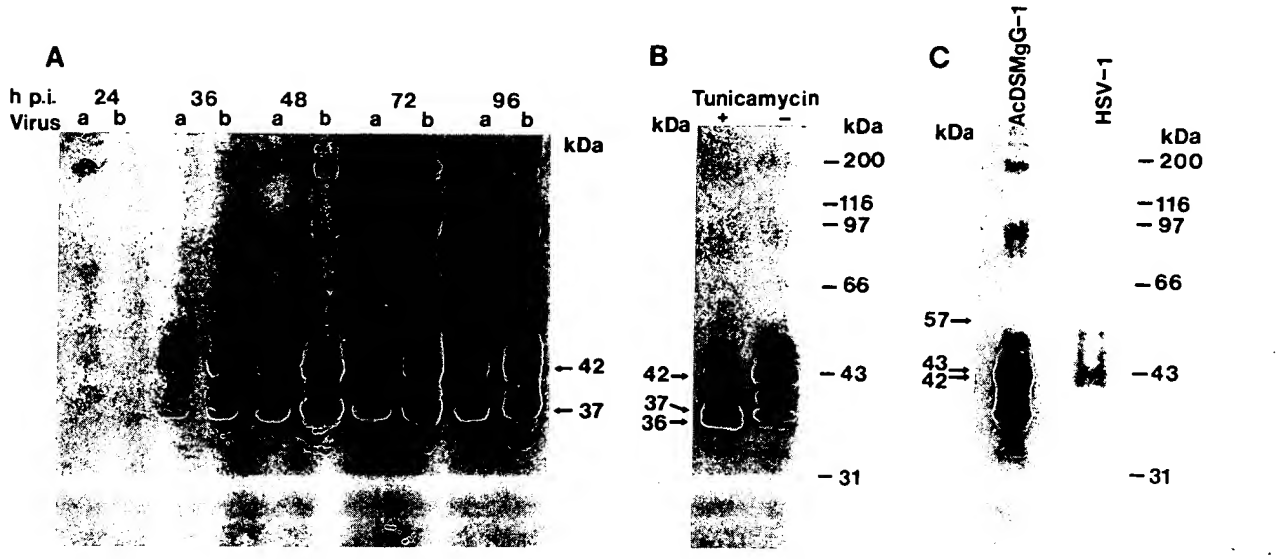

FIG. 3. Synthesis and processing of baculovirus-expressed gG-1. (A) Time course of the synthesis of gG-1 in Ac373'gG-1-infected and AcDSMgG-1-infected (lanes a and b, respectively) Sf9 cells. Cells were harvested at the indicated times, treated as those in Fig. 2A, and reacted with a HSV-1-positive human serum specimen. (B) Immunoblot of proteins extracted from Sf9 cells infected with AcDSMgG-1 and grown in the presence (+) or the absence (−) of 3 μg/ml tunicamycin from 24 hr p.i. until 54 hr p.i. Blots were reacted with gG-1-specific monoclonal antibody (H1379). (C) Immunoblot analysis of proteins extracted from Sf9 cells infected with AcDSMgG-1 and from HEp-2 cells infected with HSV-1(F); about 100-fold more infected cells of the latter were used. Myosin (200 kDa) and β-galactosidase (116.3 kDa) were used in addition to the molecular mass standards used in Fig. 2. The apparent molecular mass of bands discussed in the text (arrows) is indicated.

N-linked glycosylation (Hemming, 1982), resulted in a great reduction in the intensity of all the bands over 37 kDa apparent molecular mass, most prominently 42K, and an increase in the intensity of an otherwise very faint species of 36 kDa apparent molecular mass (36K) (Fig. 3B). The abundant 37K is of similar intensity in infected cells cultured either in the presence or in the absence of tunicamycin. In heavily loaded gels, species of 77 and 87 kDa apparent molecular mass (77K and 87K) were replaced by species of 74 and 83 kDa (74K and 83K) apparent molecular mass (data not shown).

In a comparison of baculovirus- and HSV-1-expressed gG-1, major bands with apparent molecular masses of 42 and 43 kDa (42K and 43K) and a smear between 50 and 57 kDa apparent molecular mass were found in extracts of HEp-2 cells infected with HSV-1(F) (Fig. 3C). In heavily loaded gels, the smear extended to 66 kDa apparent molecular mass (not shown).

Synthesis and processing of baculovirus-expressed gG-2

Replica immunoblots of extracts of Sf9 cells infected with AcDSMgG-2, wild-type baculovirus, or uninfected were reacted with either a HSV-2-positive and a HSV-1-negative human serum specimen or with a gG-2-specific monoclonal antibody (H1206) (Lee *et al.*, 1985) (Fig. 4A). In lanes containing extracts from AcDSMgG-2-infected cells, both antibodies reacted with bands with apparent molecular masses of 107, 118, 128, and 143 kDa (107K, 118K, 128K, and 143K). The human serum specimen reacted most strongly with 128K, and the monoclonal antibody with 118K. This difference in reactivity between the human serum and the monoclonal antibody is not a general phenomenon, inasmuch as other human serum specimens reacted most strongly with 118K (data not shown). In overloaded gels a weakly reactive protein with an apparent molecular mass of 34 kDa (34K) was detected.

We monitored expression of gG-2 in recombinant baculovirus-infected cells as a function of time after infection (Fig. 4B). Only one band (118K, open triangle) was detected at the earliest time point examined (24 hr p.i.). By 36 hr p.i. it had increased in intensity and two faint bands appeared (107K and 128K, solid triangles). From 48 hr p.i. onward, the four bands (107K, 118K, 128K, and 143K) previously seen at 100 hr p.i. (Fig. 4A) were visible, with the maximum accumulation of protein at 72 hr p.i. The weakly reactive 34K protein was first detected at 36 hr p.i.

In the electrophoretic pattern of extracts of Sf9 cells infected with AcDSMgG-2 and treated with tunicamycin, bands migrating with apparent molecular masses of 105, 110, and 120 kDa (105K, 110K, and 120K) were

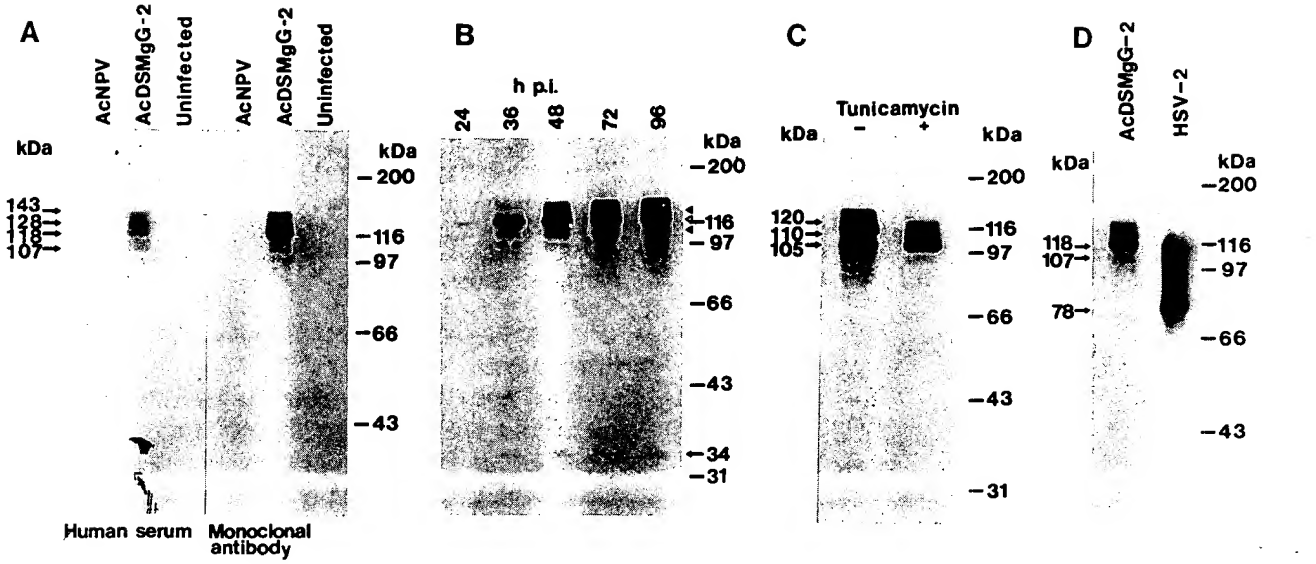

FIG. 4. Synthesis and processing of baculovirus-expressed gG-2. (A) Proteins extracted at 100 hr p.i. were separated in a 9% SDS–PAGE gel, then transferred to nitrocellulose, and reacted with the indicated antibodies. (B) Time course of the synthesis of gG-2 in AcDSMgG-2-infected Sf9 cells. Proteins extracted from cells harvested at the times indicated were treated as those in A and then reacted with gG-2-specific monoclonal antibody (H1206). (C) Immunoblot of proteins extracted from Sf9 cells infected with AcDSMgG-2 and grown in the presence (+) or the absence (−) of 3 μg/ml tunicamycin from 24 to 54 hr p.i. Blots were reacted with gG-2-specific monoclonal antibody (H1206). (D) Immunoblot analysis of proteins extracted from Sf9 cells infected with AcDSMgG-2 and from HEp-2 cells infected with HSV-2(G); about 1.8-fold more infected cells of the latter were used. Molecular mass standards were the same as those for Fig. 3. The apparent molecular mass of bands discussed in the text (arrows or triangles) is indicated.

present, whereas in untreated cells, the 107K, 118K, 128K, and 143K species were seen (Fig. 4C). In overloaded gels, a band migrating with an apparent molecular mass of 30 kDa was present in the extract of infected cells treated with tunicamycin, in contrast with the 34K species seen in untreated cells (data not shown).

In comparisons of baculovirus- and HSV-2-expressed gG-2, in the lane containing extracts from HEp-2 cells infected with HSV-2(G) (Fig. 4D), a smear of reactivity was seen ranging from an apparent molecular mass of 78 through 118 kDa with distinctive species at 78, 107, and 118 kDa apparent molecular mass. In overloaded gels, a faint band with apparent molecular mass of 36 kDa was detected (data not shown).

HSV type specificity of the reaction of human serum specimens with the baculovirus-expressed proteins

Proteins in extracts of Sf9 cells expressing the recombinant gG-1 or gG-2 were tested for reactivity with 10 different human serum specimens previously characterized by using an HSV type-specific IHA (Bernstein and Stewart, 1971). As a representative example, patterns obtained with 3 of these 10 specimens are shown in Fig. 5. Using the gG-1 37K and 42K species and the gG-2 118K species, plus either or both of the 128K and 143K species, as markers of HSV-1 and HSV-2 type-specific reaction, respectively, a serum specimen positive for HSV-1 and negative for HSV-2 by IHA, and a serum specimen positive for HSV-2 and negative for HSV-1 by IHA, each reacted in a type-specific manner in the immunoblot assay (Figs. 5A and 5B, respectively). A serum specimen weakly positive for both types of antibodies by IHA reacted with the gG-1-specific 37K and 42K species (empty triangles) in the lane of the recombinant gG-1, and reacted weakly but clearly with the gG-2 118K and 128K species (full triangles) in the lane containing recombinant gG-2 (Fig. 5C). We have seen various patterns of weak reactivity between human serum specimens and extracts of wild-type baculovirus-infected Sf9 cells as well as uninfected cells. The faint bands seen at 36 kDa apparent molecular mass in the gG-2 lanes of Fig. 5, as well as the bands seen at 170 kDa apparent molecular mass in Fig. 5C, are examples of these reactions. In no case was the reactivity of an extent or nature to lead to ambiguity in interpretation. Results obtained with the other seven serum specimens were in agreement with the IHA results (data not shown).

DISCUSSION

We constructed a transfer vector, pAcDSM, that facilitates the construction of recombinant baculovir-

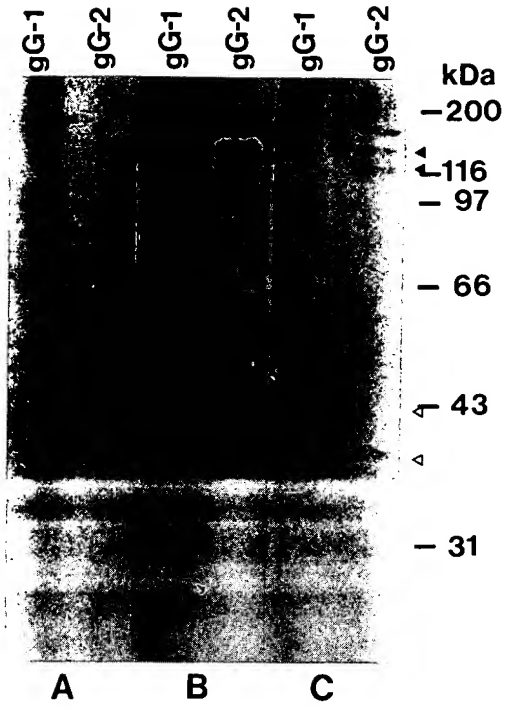

FIG. 5. Immunoblot analysis of the HSV type specificity of the reaction of human serum specimens with AcDSMgG-1- and AcDSMgG-2-infected Sf9 cell extracts. Proteins were separated by SDS–PAGE in a 11% gel, transferred to nitrocellulose, and then reacted with serum specimens known to be HSV-1-positive and HSV-2-negative (A), HSV-1-negative and HSV-2-positive (B), and having a low positive titer to both HSV-1 and HSV-2 (C). Bands considered to be diagnostic for HSV-1-specific reactivity are indicated with open triangles, and those for HSV-2 with solid triangles. Molecular mass standards were the same as those for Fig. 3.

uses containing no missing or extraneous nucleotides in the region 5′ to the translation initiation codon. No other vector reported to date allows such constructs. Although we did not compare the performance of this vector with that of recently described vectors (Matsuura *et al.*, 1987; Luckow and Summers, 1989) which more closely, albeit imperfectly, mimic the gene expression control environment of the native polyhedrin gene than earlier transfer vectors (Smith *et al.*, 1985), we did observe fourfold greater expression of gG-1 from pAcDSM than from a construct that contains 21 extraneous nucleotides in the 5′ nontranslated leader sequence. The nature of pAcDSM will allow careful dissection of the environment in the vicinity of the translation initiation codon through the construction of viruses with precise sequence modifications in this region.

Unexpectedly, in addition to the difference in the level of expression between the two gG-1-expressing recombinants, we observed a difference in the ratio of nonglycosylated precursor (37K, see below) to glycosylated product (42K), with gG-1 expressed from AcDSMgG-1 being the most efficiently processed. Inasmuch as the gG-1 coding sequence was not altered during the construction of the recombinants, these results indicate an effect of mRNA structure on protein processing efficiency. Further studies will be required to test this hypothesis.

Biosynthesis of gG-1

A scenario for the biosynthesis of gG-1 in baculovirus-infected Sf9 cells that accounts for our results is as follows: The 37K species is insensitive to tunicamycin and is likely to be the primary nonglycosylated translation product with its signal peptide uncleaved, indicating that it was not translocated to the lumen of the rough endoplasmic reticulum. The 36K species is most abundant during growth in the presence of tunicamycin and would be the nonglycosylated translation product without its signal peptide. The broad band produced by 42K, which is sensitive to tunicamycin, would be the translation product after having its signal peptide removed and being N-linked glycosylated. Species 77K, 87K, and 200K are likely to be products of further processing. In heavily loaded gels, 77K and 87K were replaced by 74K and 83K species upon treatment with tunicamycin (data not shown), indicating that the generation of the low mobility gG-1-reactive molecules is not dependent on N-linked glycosylation. The low mobility gG-reactive polypeptides are not likely to represent gG oligomers, because cell lysates were boiled in the presence of a detergent, a reducing agent, and urea prior to electrophoresis. The low abundance of the 33K species is likely to be the result of proteolytic degradation.

The biosynthesis of gG-1 in baculovirus-infected insect cells differs in several respects from its synthesis in mammalian cells infected with HSV-1- or gG-1-expressing vaccinia virus recombinants. In a direct comparison of baculovirus- and HSV-1-expressed gG-1, we observed products with apparent molecular masses of 42 and 43 kDa in HSV-1-infected cells which comigrated with 42K. It is possible that the 42- and 43-kDa species found in HSV-1-infected cells correspond to the 44- to 48-kDa species reported by others (Ackermann *et al.*, 1986) using the same strain of virus (strain F) and the same monoclonal antibody (H1379). However, the relationship between these products and the closely migrating products found in the baculovirus-infected cells is not clear. The baculovirus-expressed protein is tunicamycin sensitive and thus glycosylated, as is a product of similar size found in cells infected with a vaccinia virus/gG-1 recombinant (Sullivan and Smith, 1987), while the HSV-1 product is poorly labeled with glucosamine (Ackermann *et al.*, 1986). In addition,

a similarly sized product is synthesized in the presence of tunicamycin in HSV-1 strain HFEM-infected cells (Richman *et al.*, 1986).

Biosynthesis of gG-2

A scenario for the biosynthesis of gG-2 in baculovirus-infected Sf9 cells is as follows: 107K is the primary translation product including the signal peptide. This is based on two observations. (i) It is synthesized in the absence of tunicamycin. (ii) Since no species migrating faster than 105K were detected in cells infected in the presence of tunicamycin, 107K is inferred to not be N-linked glycosylated, is therefore likely to be unaffected by tunicamycin treatment, and is probably obscured by the other species in the gel. The 105K species is the primary translation product after cleavage of its signal peptide based on its increase in abundance in the presence of tunicamycin. The tunicamycin-sensitive 118K is the cleaved primary translation product after N-linked glycosylation. Species 128K and 143K, and 110K and 120K, synthesized in the absence or presence, respectively, of tunicamycin are possibly the products of O-linked glycosylation. The tunicamycin-sensitive 34K is likely to be the result of a proteolytic degradation.

As with gG-1, there were significant differences between the biosynthesis of gG-2 in baculovirus-infected insect cells and that in HSV-2-infected mammalian cells. In both baculovirus- and HSV-2-infected cells a tunicamycin-sensitive product of 118-kDa apparent molecular mass is synthesized. In baculovirus-infected cells, it appears that this product is processed further into higher molecular mass forms, but in HSV-2-infected cells this product is cleaved to generate species of 31 kDa (31K) (Su *et al.*, 1987) and 74 kDa (74K) (Balachandran and Hutt-Fletcher, 1985) apparent molecular mass. The 74K species is subsequently O-linked glycosylated yielding a species of 105 kDa apparent molecular mass (Balachandran and Hutt-Fletcher, 1985). The 31K species is further glycosylated to a species of 34-kDa apparent molecular mass, which is efficiently secreted from infected cells, and it is not detected by the same monoclonal antibody that detects the higher mobility products (Su *et al.*, 1987). This 34-kDa apparent molecular mass HSV-2-infected cell species differs from the similarly sized product we observed in recombinant baculovirus-infected cells, in that the baculovirus product reacts with the same monoclonal antibody as do the lower mobility products.

HSV type specificity of baculovirus expressed gG

The several differences in the synthesis and processing of gG-1 and gG-2 in insect cells, relative to that observed here and by others during infections of mammalian cells with HSV, reflect differences in protein processing mechanisms between insect and mammalian cells. Despite these differences, the recombinant proteins were recognized in a HSV type-specific manner by the 10 human serum specimens tested here. To further test the use of the baculovirus expressed proteins as substrates for serologic tests we are currently testing a set of over 80 serum specimens that had previously been characterized using gG immunodot assays (Lee *et al.*, 1985, 1986; Nahmias *et al.*, 1986). Preliminary results confirm the type specificity of baculovirus-expressed gG-1 and gG-2 (Sánchez-Martínez *et al.*, unpublished data).

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A novel glycoprotein for detection of herpes simplex virus type 1-specific antibodies

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A novel herpes simplex virus type 1 (HSV-1)-specific glycoprotein reactive with monoclonal antibody H1379 was purified by affinity chromatography. This glycoprotein, provisionally designated as gG-1, forms two sets of bands with molecular weights of 40–44,000 and 60–88,000. When used in an immunodot enzymatic assay, gG-1 reacted strongly with rabbit antisera to HSV-1, but not with sera hyperimmune to HSV-2. Specificity of the assay was further established by the lack of reactivity of convalescent sera collected from 20 patients with primary genital HSV-2 infections, and from 100 seronegative individuals. In contrast, antibodies to gG-1 were detected in 9 of 10-patients with primary HSV-1 infection, and in 63/67 patients with culture-positive, recurrent oral or genital HSV-1 infection. Reproducibility of the gG-1 immunodot assay for HSV-1 antibody detection was 96%. Serological assay with purified gG-1, done in parallel with the assay using purified gG-2 described in an earlier report, provides simple and reliable methods to detect type-specific HSV-1 and HSV-2 antibodies for sero-epidemiological studies.

herpes simplex virus, glycoprotein

Introduction

Studies with polyvalent sera and monoclonal antibodies have shown that most of the major glycoproteins specified by herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) share type-common antigenic determinants (Pereira et al., 1980; 1982; Norrild, 1980). Consequently, polyclonal antibodies prepared against viral glycoproteins of one type usually cross-react with the corresponding glycoproteins of the heterologous serotype. Until recently, it was believed that glycoprotein C of type 1 has only type-specific antigenic epitopes (Vestergaard and Norrild, 1979; Arvin et al., 1983). It has been reported, however, that cross-reactivity occurred with sera from rabbits hyperimmunized to HSV-2 (Zweig et al., 1983) and it was recently shown that the gene encoding gC-1 co-maps with a HSV-2 glycoprotein approximately 75,000 in apparent molecular weight (Zezulak and Spear, 1983). We have also observed, in a large-scale screening, that purified gC-1 reacted in a sen-

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sitive immunodot assay with sera from patients with documented primary HSV-2 infections (unpublished observations).

More recently, a glycoprotein designated as gG, 128,000 dalton in apparent molecular weight, was purified from HSV-2-infected cells (Marsden et al., 1978; Marsden et al., 1984; Roizman et al., 1984). We found this glycoprotein to be serologically type-specific and reported its use as antigen in an immunodot assay for detecting HSV-2-specific antibodies (Lee et al., 1985). Using restriction endonuclease analysis of intertypic recombinants and marker-transfer experiments, the corresponding HSV-1 glycoprotein has been tentatively identified (Ackermann et al., 1986). This protein, provisionally designated as gG-1, is specified by a gene mapped in the S component of the HSV-1 DNA collinear with the gG-2 gene in HSV-2. It was therefore of interest to study the antigenic reactivity of this new glycoprotein as a potential HSV-1 type-specific antigen for sero-epidemiological studies.

Materials and Methods

Isolation of glycoprotein gG-1

A mouse monoclonal antibody H1379-2 was used to prepare immunoaffinity columns following procedures described by Arvin et al. (1983). HEp-2 cells infected with HSV-1 (F) were harvested at 48 h post-infection and solubilized in phosphate-buffered saline (PBS, pH 7.2) containing 0.5% Nonidet P-40, 10^{-5} M *N*-tosyl-L-lysine phenylalanine ketone and 10^{-5} M *N*-tosyl-L-lysine chloromethyl ketone. The extract was clarified, adsorbed to the immunoaffinity column, and eluted with 3 M potassium thiocyanate, as previously described (Arvin et al., 1983). The eluate was concentrated 20 times by dialysis against Aquacide (Calbiochem, San Diego, CA). It was then dialyzed against PBS for 24 h at 4°C and stored in glass ampoules at −70°C until used.

Immunoblot analysis

The procedure for immunoblot analysis of HSV glycoproteins has been described elsewhere (Braun et al., 1983). Briefly, the purified glycoprotein was denatured in 2-mercaptoethanol and sodium dodecyl sulfate, electrophoretically separated in denaturing polyacrylamide gels and electrically transferred to nitrocellulose membranes. Strips of the membrane were reacted with monoclonal antibodies specific to various HSV glycoproteins, followed by horseradish peroxidase-coupled rabbit anti-mouse serum and the substrate, 4 chloro-1-naphthol.

Immunodot enzymatic assay

The purified gG-1 was used as antigen in an immunodot assay on small disks of nitrocellulose membrane in 96-well polyvinyl chloride plates. One microliter of diluted antigen was immobilized on each disk. Details of the assay procedure have been reported elsewhere (Lee et al., 1985). Each serum was tested in duplicate at 1:50 dilution. To ensure standardization of the assay, the following serum controls

were included in each assay: HSV-1 serum pool diluted 1:800 and 1:3200; HSV-2 serum pool diluted 1:50 and 1:200, and a negative serum pool diluted 1:50. Results were accepted only when both dilutions of the HSV-1 serum pool were positive and all the other controls were negative.

Sera

Hyperimmune rabbit sera to HSV-1 (Shealey or VR3) and HSV-2 (MS) were prepared, as detailed elsewhere (Lee et al., 1985). Convalescent sera obtained previously (Nahmias et al., 1969) from rabbits with keratitis induced by ocular inoculation of HSV-1 (VR3 or Shealey) and HSV-2 (MS) were also included for testing.

Human sera were collected from individuals with clinically manifest and culturally proven HSV-1 and/or HSV-2 infections. Viral isolates from these patients were typed by restriction endonuclease analysis of viral DNA (Buchman et al., 1980) and/or direct immunofluorescence using type-specific monoclonal antibodies (Pereira et al., 1982). A screening enzyme-linked immunosorbent assay (ELISA) with a pool of Triton X-100-extracted antigens from HSV-1- and HSV-2-infected HEp-2 cells (Coleman et al., 1983) detected and quantitated total HSV antibodies in these sera. Primary HSV infections were defined as those in which no pre-existing antibody was detected in the acute sera, with HSV antibodies later demonstrated in the convalescent phase. Recurrent cases are those in which the individual had a history of clinically recurrent oral or genital lesions and HSV antibodies were detected at the time of the recurrence. In addition, 100 HSV seronegative sera (as determined by the screening ELISA) collected from healthy donors were included as negative controls. Two hundred and five sera collected from on going epidemiological studies in a health maintenance organization were used, in a blind manner, to test the reproducibility of the gG-1 immunodot assay. For standardization of the gG-1 serological assay, 10 convalescent sera from patients with primary HSV-2 infection were pooled and used as HSV-2 control serum, 20 sera from patients with HSV-1 infections were pooled as HSV-1 control, and 20 sera from healthy donors negative in the screening ELISA, were pooled as negative control.

Results

Characterization of glycoprotein gG-1

To determine whether the novel type-1 glycoprotein was antigenically distinct, the eluate from the H1379 immunoaffinity column was electrophoretically separated in denaturing polyacrylamide gels, electrically transferred to nitrocellulose and compared with affinity purified HSV-1 glycoproteins gB, pgC and gD by reactivity with monoclonal antibodies to each glycoprotein. In this series of experiments, illustrated in Fig. 1, protein bands eluted from the H1379 affinity column migrated with apparent molecular weights of 44,000 to 68,000. The monoclonal antibody failed to react with an eluate from extracts of mock-infected cells adsorbed to the H1379 column. It is notable that the electrophoretic properties of

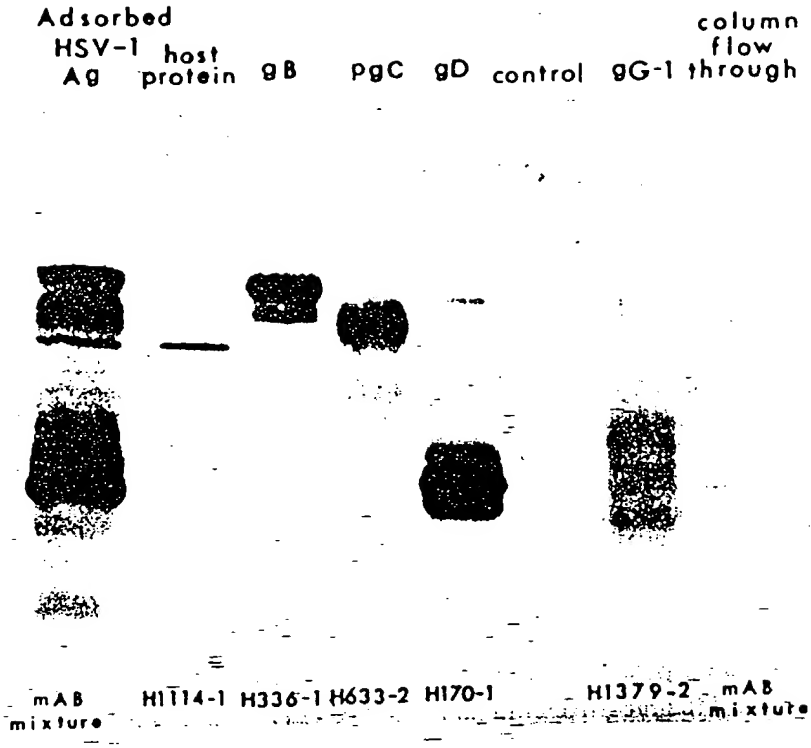

Fig. 1. Immunoblot analysis of immune reactivity of glycoproteins purified from extracts of HSV-1-infected cells. Monoclonal antibodies used for immune reactions with affinity column eluates are shown at bottom of each lane. Purified glycoproteins are designated at top of each lane. Infected cell extracts adsorbed to the column are shown in the left lane. Column flow-through is shown in the right lane.

gG-1 differ from those of glycoproteins gB-1, pgC-1 and gD-1. The difference in antigenicity is further illustrated in Fig. 2. When the purified gG-1 was used as antigen in immunodot assays with mouse monoclonals specific for the various HSV-induced glycoproteins, only H1379, specific for gG-1, reacted.

Standardization of immunodot enzymatic assay with purified gG-1

Serial two-fold dilution of gG-1 was tested against 1:50 dilution of 10 convalescent sera collected from individuals with primary HSV-1 infections. The optimum antigen (gG-1) dilution, as judged by the intensity of the color reaction, was found to be 1:64. Reactivity significantly diminished when the antigen was used either at low dilutions ($\leq$1:16) or high dilutions ($\geq$1:240).

Specificity and sensitivity of purified gG-1 in immunodot assay

Specificity was first evaluated using rabbit immune sera. All sera collected from 7 rabbits infected by the ocular route with HSV-1 (Shealey) and 3 rabbits hyperimmunized with HSV-1 (VR3) reacted strongly with gG-1. In contrast, no reac-

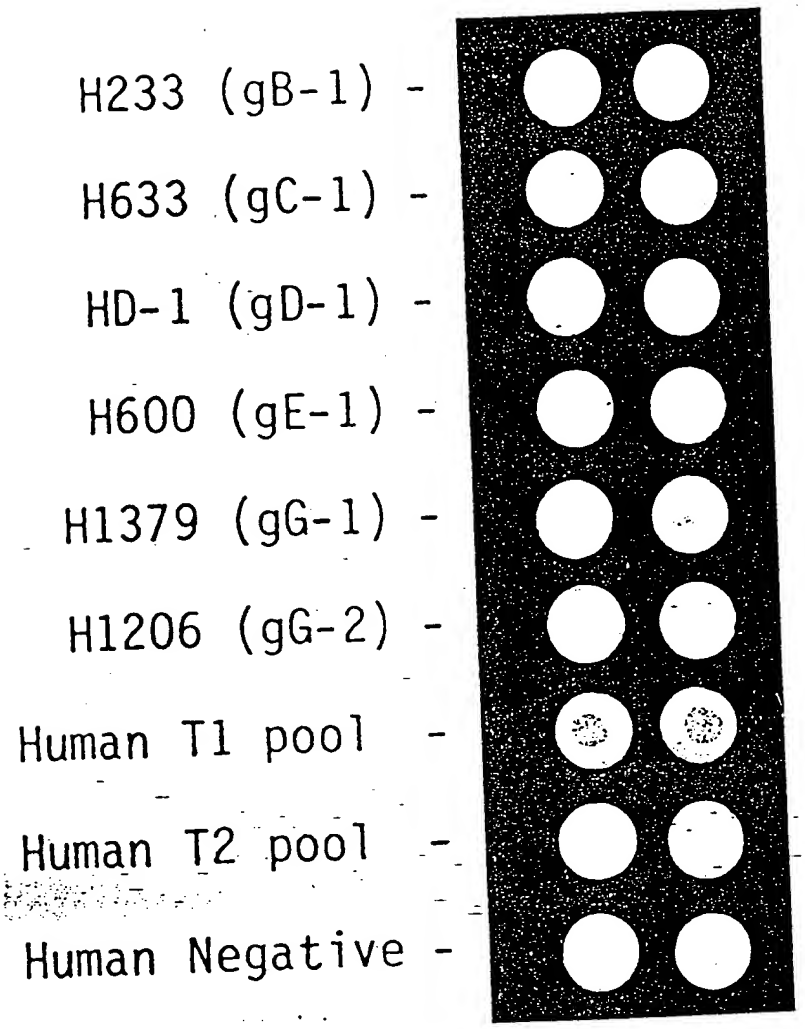

Fig. 2. Immunodot analysis of immune reactivity of purified gG-1. Monoclonal antibodies, with their specificity in parentheses, and human sera used for immune reactions are shown in the left column. Human T1 pool: pool of 20 sera collected from patients with HSV-1 infection; Human T2 pool: pool of 10 convalescent sera from patients with primary HSV-2 infection; Human Negative: pool of 20 sera from healthy donors, negative in the screening ELISA.

tivity was detected in sera from 9 rabbits hyperimmunized or infected with HSV-2 (MS). Sera collected from 2 pre-immune rabbits, and from 2 rabbits hyperimmunized with HEp-2 cells and fetal calf serum were also negative.

When tested with human sera (Table 1), gG-1 reacted with 9 of 10 convalescent sera collected from patients who had primary HSV-1 infections. Negative results were obtained for all of the convalescent sera collected from 20 patients with primary genital HSV-2 infection. Moreover, when 100 sera identified as HSV sero-

TABLE 1

Specificity of immunodot gG-1 antibody assay

Group tested	No. tested	No. positive for HSV antibody*	No. positive for gG-1 antibody
Primary HSV-1	10	10	9
Primary HSV-2	20	20	0
HSV seronegatives (screening test)	100	0	0

* Detected in ELISA using, as antigen, Triton X-100 extract of HEp-2 cells infected with HSV-1 and HSV-2 viruses (Coleman et al., 1983).

negative by a screening ELISA (Coleman et al., 1983) were tested, none reacted with gG-1.

The sensitivity of the gG-1 immunodot assay was evaluated by testing human sera collected from patients in whom HSV-1 had been isolated (Table 2). Antibody to gG-1 was demonstrated in 94% of the 67 patients with recurrent labial or genital HSV-1 infections. When acute and convalescent sera obtained from 10 patients with primary HSV-1 genital infection were tested, only 1 of 7 sera collected within 10 days after onset of symptoms reacted with gG-1, whereas 9/10 sera collected thereafter were positive.

Reproducibility

To test the reproducibility of the immunodot assay with purified gG-1, 205 sera were repeat-tested as coded samples. There was concordance in 96% of the results between the second and the first tests. The dissimilar results were obtained primarily with sera having very low HSV antibody titers (as determined in the screening ELISA).

Discussion

The glycoprotein isolated with monoclonal antibody H1379, provisionally designated gG-1, appears to be antigenically distinct from gB, gC, gD and gE of HSV-

TABLE 2

Sensitivity of immunodot gG-1 antibody assay

Patients with HSV-1 isolated	No. cases	No. (%) with HSV antibodies*	No. (%) with gG-1 antibodies
Recurrent cases	67	67 (100%)	63 (94%)
Primary cases (days after onset)			
≤ 10	7	2 (29%)	1 (14%)
≥ 11	10	9 (90%)	9 (90%)

* Detected in ELISA using, as antigen, Triton X-100 extract of HEp-2 cells infected with HSV-1 and HSV-2 viruses (Coleman et al., 1983).

1 (Fig. 2). It is probably identical with the new glycoprotein of HSV-1 of approximately 59,000 MW, also mapping in the short DNA segment, recently reported by Richman et al. (1986). The apparent wide range of molecular weights of proteins reactive with the monoclonal antibody H1379, as demonstrated in the Western blot analysis (Fig. 1), presumably represents components of gG-1 at various stages of maturation. Unlike glycoprotein C of herpes simplex type 1 which cross-reacts with sera from rabbits hyperimmunized to HSV-2, we have found the gG-1 to be type-specific with both human and hyperimmune rabbit sera. A high degree of sensitivity, specificity and reproducibility has been demonstrated using gG-1 in an immunodot enzymatic assay. Only a small amount of the purified antigen (2 μl of the appropriate dilution) is required in the immunodot assay, as compared to 50 times that amount in an ELISA (Coleman et al., 1983). Together with the gG-2 immunodot assay reported earlier (Lee et al, 1985), this gG-1 assay provides a simple and reliable means to detect HSV-1 and HSV-2 type-specific antibodies independently. We have also observed a large number of human sera, with titer >1:12800 which reacted with only gG-1 or gG-2 but not both antigens. This observation further establishes the specificity of the assays even in the presence of high-titer heterotypic antibodies.

Since HSV-1 is usually associated with infections of the upper body, while HSV-2 is more often found to infect genital sites (Nahmias et al., 1981), assays that can distinguish antibodies against these viruses are of particular epidemiological importance. Identification of patients with antibodies to either one or both HSV types should be of significant help in sero-epidemiological studies to define the prevalence and incidence of HSV-1 and HSV-2 infections in different populations (e.g., Keyserling et al., 1985; Gibson et al., 1985; Lossick et al., 1985), as well as possible relations of genital herpes to cervical cancer (Nahmias et al., 1985).

Acknowledgements

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Detection of Herpes Simplex Virus Type 2-Specific Antibody with Glycoprotein G

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A recently described herpes simplex virus (HSV) type 2 (HSV-2)-specific glycoprotein (gG-2) was purified on an immunoaffinity column prepared with monoclonal antibody. This purified antigen was used in an immunodot enzymatic assay on nitrocellulose paper for the detection of HSV-2 antibodies in human serum. The test was very sensitive in that HSV-2 antibodies were detected in the convalescent sera of 132 of 134 patients with recurrent genital infections in which HSV-2 had been isolated earlier. Antibodies to gG-2 were detected in 17% of sera obtained within 10 days after the onset of a primary HSV infection and in 95% of sera obtained more than 10 days after onset. The specificity of the immunodot assay was demonstrated by testing sera from 245 HSV-seronegative adults, 344 children, 29 nuns, and 13 patients with primary genital HSV-1 infections. None of these 631 sera was reactive with the gG-2 antigen. When compared with a microneutralization test, the immunodot assay was found to be more specific in detecting HSV-2 antibodies. Reproducibility of the gG-2 assay, obtained by retesting 391 sera, was 95%. Thus, this assay has the sensitivity, specificity, and reproducibility necessary for the measurement of HSV-2 antibodies in seroepidemiological studies.

Since the demonstration of two antigenic types of herpes simplex virus (HSV), type 1 (HSV-1) and HSV-2 (7, 14), numerous methods have been devised to differentiate these two closely related viruses. The recent ability to determine restriction endonuclease digestion patterns of herpesvirus DNA (2) and to use monoclonal antibody in various immunological assays (11, 12) has enabled the typing of virus isolates with consistent accuracy. However, the delineation of HSV-1 and HSV-2 type-specific antibodies in humans, of particular import for definitive seroepidemiological investigation, has remained difficult because of the extensive antigenic cross-reactivity between the two virus types (15).

The development of monoclonal antibodies for the two HSV types has made it possible to isolate specific HSV proteins (1, 3) which could be applied to more specific antibody assays. It was of particular interest to explore the usefulness for type-specific antibody testing of a recently described HSV-2-specific glycoprotein, gG-2 (13). This glycoprotein is specified by a gene mapping in the S component of the genomic DNA of HSV-2 (4, 13). In this report, we describe the evaluation of an HSV-2 type-specific enzyme-linked immunodot serological assay based on affinity-purified gG-2 as antigen.

MATERIALS AND METHODS

Sera. (i) Animals. Hyperimmune rabbit sera to HSV-1 (Shealey and VR3 strain) and HSV-2 (MS strain) were prepared by four biweekly intravenous injections of about 10^7 PFU of virus into male New Zealand albino rabbits. The sera were collected 2 weeks after the final injection. Convalescent sera obtained previously (8) from 13 rabbits with keratitis induced by ocular inoculation of HSV-1 (VR3 or Shealey strain) and HSV-2 (MS strain) were also included for testing.

(ii) Humans. Sera were obtained from individuals with clinically manifest and culturally proven HSV-1 or HSV-2 infections. Viral isolates from these patients were typed by restriction endonuclease analysis of viral DNA (2) or by direct immunofluorescence with type-specific monoclonal antibodies (11) or by both. A screening enzyme-linked immunosorbent assay (ELISA) with a pool of Triton X-100-extracted antigens from HSV-1- and HSV-2-infected HEp-2 cells (3) detected and quantitated total HSV antibodies in these sera. Primary HSV infections were defined as those in which no preexisting antibody was detected in the acute sera, with HSV antibodies later demonstrated in the convalescent phase. Recurrent cases were defined as those in which the individual had a history of clinically recurrent genital lesions and in which HSV antibodies were detected at the time of the recurrence.

A collection of 344 sera from children between 1 and 10 years of age was tested. The ages were chosen because transplacental antibodies would have been lost by 1 year of age, and an HSV-2 infection between 1 and 10 years would either have been a result of a neonatal infection or an unusual postnatally acquired HSV-2 infection. The 29 sera from nuns, which had been tested earlier with a microneutralization assay (9), were included because HSV-2 infections would be unlikely in this population. In addition, for purposes of evaluating the reproducibility of the assay, 391 sera collected from ongoing epidemiological studies in a health maintenance organization were used.

For standardization of the gG-2 serological assay, 10 convalescent sera from patients with primary HSV-2 infections were pooled and used as HSV-2-positive controls, 20 high-titered sera from patients with HSV-1 infections were pooled and used as HSV-1-positive controls, and 20 sera from healthy donors, negative in the screening ELISA (3), were pooled and used as negative controls.

Antigen preparation. Two mouse monoclonal antibodies, H966 and H1206, were used to prepare immunoaffinity columns for the isolation of gG-2 from HEp-2 cells infected with the G strain of HSV-2. The properties of the H966 antibody and the detailed procedure for the immunoaffinity purification of HSV proteins have been described elsewhere

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TABLE 1. Sensitivity of immunodot gG-2 antibody assay

Patients with HSV-2 isolated	No. of cases	No. (%) with gG-2 antibodies
Recurrent cases	134	132 (99)
Primary cases (days after onset)		
≤10	12	2 (17)
11–20	15	14 (93)
>20	5	5 (100)

(1, 13). It was later found that immunoaffinity columns prepared with monoclonal H1206 antibody, also specific for gG-2, gave a higher yield of antigen. The antigens prepared with these two monoclonal antibodies were identical, and both were identified as glycoprotein G by Western blot analysis.

Immunodot enzyme assay. An immunodot enzyme assay with purified gG-2 was used for conservation of antigens. Thus, instead of the 200 μl needed for duplicate testing in a standard ELISA (e.g., reference 3), 2 μl of the diluted antigen could be used in the immunodot assay. Small disks of nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) were prepared and deposited directly into 96-well polyvinyl chloride plates (Dynatech Laboratories, Inc., Alexandria, Va.) with a 96-hole punch. The detailed design and operation of this punch will be described separately (H. Keyserling, manuscript in preparation). The nitrocellulose disks in the plates were washed once with distilled water and left to dry completely at ambient temperature (a.t.). The gG-2 antigen preparation was diluted in Tris-buffered saline (TBS; pH 7.2). Optimal dilutions of antigens were determined in block titrations with a known HSV-2-positive serum pool and were 1:16 for the H966 lot and 1:64 for the H1206 lot. Onto the center of each disk, 1 μl of the diluted antigen was delivered with a microsyringe fitted with a repeating dispenser (The Hamilton Co., Reno, Nev.). After drying at a.t. overnight, the disks in the 96-well plates were washed once with TBS (10 min) and incubated at a.t. for 30 min on a rotating platform (TekPro; American Hospital Supply Corp., Evanston, Ill.) with 100 μl of TBS supplemented with 5% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.). The buffer was then removed, and 100 μl of test sera, diluted 1:50 in TBS-BSA, was added to duplicate wells. The plates were placed on the rotating platform and left to incubate overnight at a.t. The wells were washed with TBS (three times for 10 min with rotation) and reblocked with 100 μl of TBS-BSA for 30 min. Goat anti-human immunoglobulin G peroxidase conjugate (Miles Laboratories, Inc., Elkhart, Ind.) was diluted in TBS-BSA (1:1,000), and 100 μl was added to each well. Incubation took place at a.t. for 1 h on the rotating platform. After the plates were washed as before, 100 μl of substrate solution (6 mg of 4-chloro-1-naphthol dissolved in 2 ml of methanol mixed with 10 ml of TBS and 5 μl of 30% H_2O_2) was added to each well. The reaction was stopped after 15 min by removing the substrate solution and washing the plate twice with distilled water. The nitrocellulose disks were read after drying overnight at a.t. in the dark. A positive reaction was indicated by the presence of a bluish-purple dot in the center of the disk. Each assay was read by two individuals; results in the duplicate wells needed to be agreed to by both readers or the test was repeated. The following serum controls were included in each assay: HSV-2-positive serum pool diluted 1:400 and 1:1,600; HSV-1-positive serum pool diluted 1:50 and 1:200, and a negative serum pool diluted 1:50. To ensure standardization of the assay, results were accepted only when both dilutions of the HSV-2 serum pool were positive and all the other controls were negative. No more than 5% of the tests were repeated because of a reading discrepancy, and <1% were repeated because of unacceptable controls.

ELISA for total HSV antibodies. The details of the screening ELISA have been published elsewhere (3).

Microneutralization test. The technique of the microneutralization assay for detecting type-specific HSV antibodies has been described previously (8, 9).

RESULTS

Reactivity of gG2 with rabbit sera. Sera from nine rabbits hyperimmunized with HSV-1 (Shealey or VR3 strain) and convalescent sera from 13 rabbits with HSV-1 keratitis were tested with antigens prepared from the monoclonal H1206 column. None reacted with gG-2 in the assay. In contrast, all six sera from rabbits immunized or infected ocularly with HSV-2 (MS strain) yielded positive reactions. Sera from nonimmunized rabbits or from those hyperimmunized with HEp-2 cells and fetal calf serum were also negative in the assay.

Reactivity of gG-2 with human sera. Sensitivity and specificity studies with human sera from 13 primary HSV-1 infections and 14 primary HSV-2 infections demonstrated results identical to those obtained with gG-2 prepared from the H966 and the H1206 columns. The data with human sera obtained by using gG-2 prepared from the H966 column are noted below.

(i) Sensitivity. The sensitivity of the assay for HSV-2 antibodies was evaluated by using sera of 134 patients with recurrent genital herpes in which HSV-2 had been isolated (Table 1). Antibody to gG-2 was demonstrated in 99% of sera collected from 134 recurrent cases. When sera obtained from 32 patients with primary HSV-2 infections were tested, antibody to gG-2 was detected in only 17% of sera obtained within 10 days of onset; 95% of sera obtained thereafter were positive (Table 1).

(ii) Specificity. The specificity of the assay was evaluated by testing sera from individuals in several special categories (Table 2). Direct evidence of specificity was obtained by demonstrating no reactivity to gG-2 with all 13 of the convalescent sera from patients with primary genital HSV-1 infections. Moreover, none of the 245 sera identified as seronegative to HSV by the screening ELISA (3) reacted with gG-2. Of the 344 sera from children 1 to 10 years of age, 167 reacted with the screening ELISA (3) and two reacted in the gG-2 immunodot enzyme assay. On analysis of the clinical records of these two children, it was found that one serum sample had been included by mistake in that it had been obtained from a 4-month-old infant who had received

TABLE 2. Specificity of immunodot gG-2 antibody assay

Group tested	No. positive/ total tested
Primary genital HSV-1	0/13
Seronegative (screening test)	0/245
Children	2[a]/344
Nuns	0/29

[a] A 4-month-old child had also received gamma globulin earlier, and a 9-year-old girl had vulvar lesions compatible with genital herpes.

gamma globulin a few days earlier. The other serum sample was from a 9-year-old girl who had been hospitalized 2 weeks earlier with vulvar lesions compatible with genital herpes. Of interest is that all 29 sera from nuns were negative by the gG-2 assay, 19 having reacted in the screening ELISA.

As an indirect measure of the specificity of the gG-2 antibody assay, we compared the results of this test with those obtained earlier with a microneutralization assay in 54 sera (9). The results agreed with those observed in all sera previously reported as seronegative or as possessing only HSV-1 antibodies (Table 3). However, 8 of 23 sera previously noted to be positive for type 2 antibody by the microneutralization test were found to be negative by the gG-2 immunodot assay.

(iii) Reproducibility. We selected 291 sera, collected at a health maintenance organization, which were positive for gG-2 antibody. On repeat of the test, 273 (94%) were again found to be positive. When 100 sera collected consecutively at the health maintenance organization were repeated as coded samples, 97 were found to show results (positive or negative) similar to those found in the first testing.

TABLE 3. Comparison of immunodot gG antibody assay with microneutralization test

Antibody found by microneutralization test	No. of results by gG-2 antibody assay	
	Positive	Negative
None		14
HSV-1		17
HSV-2	15	8

DISCUSSION

Since HSV-2 is the type most usually associated with genital or neonatal infections (6), a serological assay that can detect HSV-2 antibodies would be of particular epidemiological assistance. However, because of the existence of many common antigens in HSV-1 and HSV-2 (15), specificity of the assay has been a major problem. Cross-absorption of patient sera with HSV-1 antigens (e.g., reference 10) has limitations because sera with high-titer antibodies to HSV-1 occur frequently and complete absorption of cross-reacting antibody is often very difficult. Moreover, the procedure of cross-absorption may deplete homologous antibody titers. Neutralization potency measurements (8) or determinations of HSV-1/HSV-2 ratios in microneutralization or other immunological assays (10, 16) are most accurate in sera that have antibody to either HSV-1 or HSV-2. In patients with both HSV-1 and HSV-2 infections, interpretation of the antibody type(s) can become difficult. For instance, 8 of 23 sera positive for HSV-2 by the microneutralization assay were later found to be negative by the gG-2 assay (Table 3). In view of the high sensitivity of the gG-2 assay (99%; Table 1), it is likely that the HSV-2 antibodies detected in the microneutralization test represent false-positive results.

With the availability of mouse monoclonal antibodies, it has become possible to purify HSV-2 proteins that fail to express type-common antigenic determinants detectable in serological assays. The use of gG-2, purified from extracts of HSV-2-infected cells, enabled us to develop an assay of high sensitivity, specificity, and reproducibility. Moreover, the immunodot assay is suitable for screening large numbers of sera because it requires a small amount of purified glycoprotein. Purified gG-2 retains antigenicity at −70°C for over 1 year when stored in glass but not plastic ampoules. We have also noticed that BSA from different sources could influence the results of the gG-2 assay (some batches of BSA caused significant reductions in the sensitivity of the assay). We overcame this problem by testing different batches of BSA from several sources and arranged to have a large stock of the optimal batch from its supplier.

Any serologic assay for HSV-2-specific antibodies requires thorough evaluation because of the epidemiological implications, e.g., the relation of genital herpes with cervical cancer (5, 17). At the individual level, false-positive results might lead to great problems, e.g., improper medical management for pregnant women or undue psychological trauma in patients and their consorts. The gG-2 serological assay for detecting HSV-2 antibodies has therefore been subjected to assiduous controls for sensitivity, specificity, and reproducibility. The results obtained indicate its usefulness for more definitive measurement of HSV-2 antibodies than has heretofore been available in seroepidemiological studies.

ACKNOWLEDGMENT

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Baculovirus Expression Vectors: the Requirements for High Level Expression of Proteins, Including Glycoproteins

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SUMMARY

The requirements for high level expression of three foreign proteins using the polyhedrin gene promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV, Baculoviridae) have been investigated. In *Spodoptera frugiperda* cells infected with the appropriate recombinant baculoviruses, the synthesis of the two S RNA coded genes of lymphocytic choriomeningitis virus (LCMV; i.e. the nucleoprotein, N, and glycoprotein precursor, GPC), or the haemagglutinin gene of influenza A virus, appears to be related to the degree of integrity of the 5′ upstream sequence of the polyhedrin gene. No effect on the level of N protein expression was detected when all the polyhedrin gene coding sequences or some of the immediate 3′ downstream sequences were deleted. Using the most efficient expression viruses derived from a new transfer vector, pAcYM1, it has been estimated that LCMV N protein represented approximately 50% of the total cellular protein, an observation consistent with the presence of numerous inclusion bodies in the cytoplasm of infected cells. For recombinant viruses derived from the pAcYM1 transfer vector containing the LCMV GPC gene, the level of synthesis of the arenavirus glycoprotein was equivalent to approximately 20% of the cellular protein. Thin sections of cells infected with the GPC recombinant revealed a highly vacuolated cytoplasm.

INTRODUCTION

The 3′ half of the S RNA species of the WE strain of lymphocytic choriomeningitis virus (LCMV; Arenaviridae) codes for the viral nucleoprotein (N) in its viral-complementary sequence. The 5′ half of the S RNA codes for glycoprotein precursor (GPC) protein in the viral-sense sequence (Romanowski & Bishop, 1985; Romanowski *et al.*, 1985). The sequences of the two genes do not overlap and in infected mammalian cells there are discrete subgenomic mRNA species that are involved in the synthesis of the two viral proteins, as shown previously for Pichinde arenavirus (Auperin *et al.*, 1984*a*, *b*). The LCMV S RNA has been cloned into DNA, inserted into transfer vectors derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV), and used, depending on the LCMV S DNA orientation, to make recombinant baculoviruses that directed the synthesis of the respective arenavirus proteins in infected insect cells derived from *Spodoptera frugiperda* (Matsuura *et al.*, 1986). Expression of the haemagglutinin (HA) protein of influenza A virus (A/PR/8/34 strain) in *S. frugiperda* cells has previously been described using recombinant baculoviruses (Possee, 1986; Kuroda *et al.*, 1986).

The baculovirus expression system was chosen in view of the reports of high levels of foreign gene expression obtained with the viral polyhedrin promoter (Smith *et al.*, 1983, 1985; Miyamoto *et al.*, 1985; Maeda *et al.*, 1985). However, using the available AcNPV-based pAcRP transfer vectors to produce recombinant baculoviruses (Possee, 1986; Matsuura *et al.*, 1986), the highest level of expression of the LCMV genes, or influenza virus HA, as judged by analysis of stained proteins respresenting extracts of infected cells, did not reach that of the polyhedrin protein expressed by AcNPV (unpublished data). Similar results have been obtained with the

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Fig. 1. Transfer vectors compared to AcNPV. The 5′-proximal leader sequences of various transfer vectors used to produce recombinant baculoviruses are given by comparison with the AcNPV sequence (Hooft van Iddekinge *et al.*, 1983; Howard *et al.*, 1986). The *Bam*HI linker sequences are underlined. The numbering system is based on the AcNPV polyhedrin ATG initiation codon representing residues +1, +2, +3 with upstream nucleotides having negative number assignments. The amino acids coded by the AcNPV polyhedrin gene are indicated under their respective codons.

pAc373 vector of Smith *et al.* (1983). A variety of reasons may account for these observations, e.g. a requirement for the deleted upstream sequences, or for the deleted polyhedrin gene coding sequences, or its product, or reduced transcription with large inserts of foreign DNA, or foreign gene product inhibition, etc. In order to determine the reason for the reduced levels of synthesis of foreign genes, an investigation of the requirements for optimal expression has been initiated. On the basis of results obtained for influenza virus HA and the LCMV N and GPC proteins using a variety of new AcNPV vectors, the level of foreign gene expression appears to be related to the integrity of the 5′ non-coding region of the polyhedrin gene rather than to the polyhedrin coding or immediate 3′ downstream sequences.

METHODS

Viruses and cells. AcNPV and recombinant virus stocks were grown and assayed in confluent monolayers of *S. frugiperda* cells in medium containing 10% foetal bovine serum according to the procedures described by Brown & Faulkner (1977). On occasion virus stocks were obtained using spinner cultures of *S. frugiperda* cells. The WE strain of LCMV was grown in BHK-21 cells and titrated in Vero cells (Romanowski & Bishop, 1983).

DNA manipulations and construction of DNA clones. Plasmid DNA manipulations were carried out essentially as summarized by Maniatis *et al.* (1982). Restriction enzymes, T4 DNA ligase and the Klenow large fragment of DNA polymerase were purchased from Biolabs (Beverly, Mass., U.S.A.). Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim.

Insertion of LCMV S or influenza HA viral DNA into transfer vectors with partial 5′ upstream sequences and a modified polyhedrin gene. A transfer vector plasmid containing a 7·3 kb AcNPV *Eco*RI I fragment in pUC8 was initially modified in a manner analogous to that described by Smith *et al.* (1983) by removal of the pUC8 *Bam*HI site (modified pUC8; Possee, 1986) followed by replacement with a *Bam*HI linker of the polyhedrin gene ATG codon plus a number of nucleotides preceding and approximately 170 nucleotides following the ATG codon (see Possee, 1986). As demonstrated by Howard *et al.* (1986) the polyhedrin gene −1 nucleotide (Fig. 2) differs from that described by Smith *et al.* (1983). The transfer vectors (pAcRP1 to 22, Fig. 1, 2) have a single *Bam*HI restriction site for insertion of new genes and contain some two-thirds of the coding region of the polyhedrin gene as well as all of the 3′ downstream sequences. In order to obtain derivatives of these transfer vectors containing foreign DNA inserts, plasmid DNA was digested to completion with *Bam*HI, dephosphorylated and ligated to

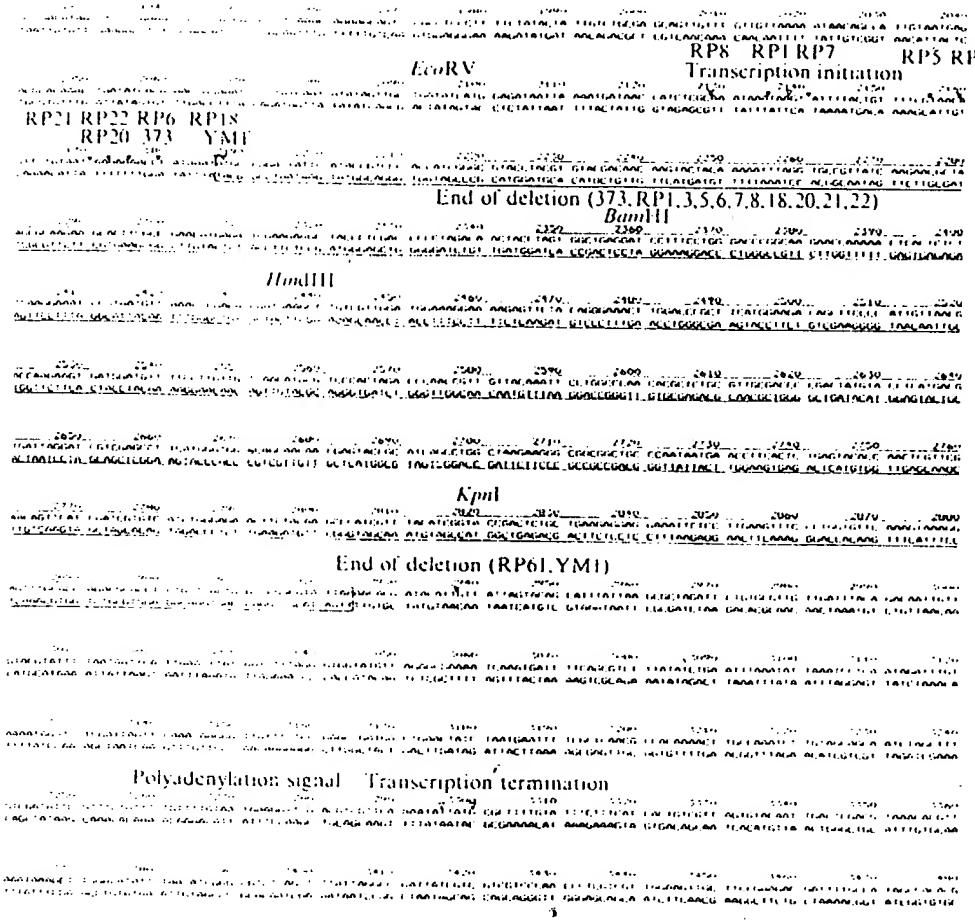


Fig. 2. The AcNPV transfer vector deletions are shown relative to the AcNPV polyhedrin gene, its transcription initiation site (open triangle; Howard *et al.*, 1986), polyadenylation and transcription termination signals (open triangles), and flanking sequences. The deletion sites of the various vectors used in the analyses are indicated by filled triangles (see Fig. 1). Particular restriction enzyme sites used for plasmid constructions are also indicated.

DNA recovered from appropriate plasmids containing viral inserts as described previously (Possee, 1986; Matsuura *et al.*, 1986). After transformation and screening with the appropriate nick-translated DNA, recombinant plasmids were obtained that were subsequently characterized by restriction enzyme and sequence analyses (Maxam & Gilbert, 1980).

Construction of a transfer vector, pAcRP61, lacking the carboxy-proximal polyhedrin coding sequences and 13 downstream (3′) nucleotides. Plasmid DNA representing the transfer vector pAcRP6 (Matsuura *et al.*, 1986; Possee, 1986), i.e. lacking the polyhedrin ATG codon, seven preceding nucleotides and some 170 nucleotides following the ATG codon, see Fig. 1, 2) was digested to completion with *Kpn*I then partially with the exonuclease Bal31, repaired with the Klenow fragment of DNA polymerase, dephosphorylated and ligated to a *Bam*HI linker. After digestion with *Bam*HI, the resulting DNA was circularized and used to transform *Escherichia coli*. Among the transformants a plasmid, designated pAcRP61, was identified that lacked the carboxy-proximal coding sequences of the polyhedrin gene (see Fig. 2) as well as 13 downstream nucleotides. A schematic representation of the derivation of pAcRP61 is provided in Fig. 3.

Construction of transfer vector pAcYM1 with all the upstream sequences of the polyhedrin gene including the A of the initiating ATG codon but lacking the rest of the polyhedrin coding sequences and 13 downstream nucleotides. The modified pUC8-based plasmid containing the *Eco*RI I fragment of AcNPV (see above) was digested to completion with *Bam*HI, the largest DNA fragment was recovered, digested partially with Bal31, repaired with

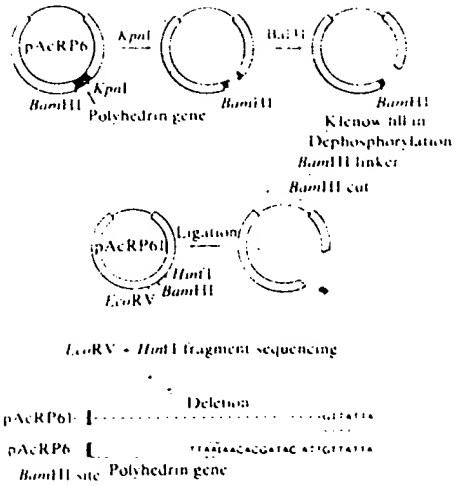


Fig. 3

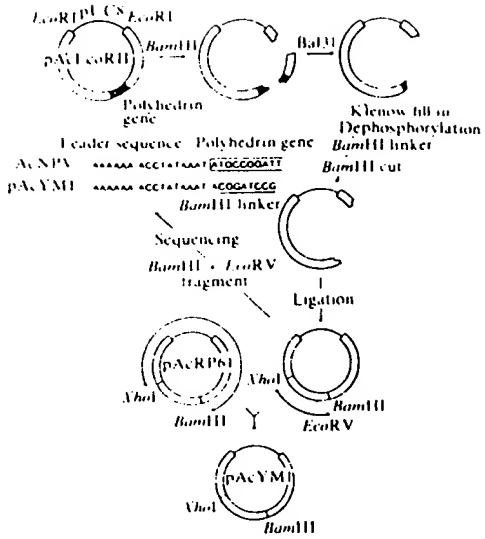


Fig. 4

Fig. 3. Schematic diagram of the construction of the transfer vector pAcRP61 as described in Methods.

Fig. 4. Schematic diagram of the construction of the transfer vector pAcYM1 as described in Methods.

the Klenow fragment of DNA polymerase, dephosphorylated and ligated to a *Bam*HI linker. After digestion with *Bam*HI, the DNA was circularized and used to transform *E. coli*. Among the transformants a plasmid was obtained that sequence analyses indicated possessed the A of the initiating ATG codon and all of the upstream sequences adjacent to the polyhedrin gene. By recovering the small *Xho*I to *Bam*HI restriction fragment from this plasmid and ligating it to the large *Bam*HI to *Xho*I fragment recovered from pAcRP61, followed by transformation (etc.) a transfer vector designated pAcYM1 was produced, containing all the upstream sequences of the polyhedrin gene and the A of the initiating ATG codon but lacking the rest of the polyhedrin coding sequences and 13 3′ downstream nucleotides as indicated in Fig. 1.2. The derivation of the pAcYM1 vector is shown in schematic form in Fig. 4

Insertion of individual LCMV S-coded genes (N or GPC) into transfer vectors. Plasmid pAcRP6YN2 (Matsuura *et al.*, 1986) was digested to completion with *Hin*dIII, the smallest LCMV DNA fragment recovered and digested partially with *Hae*II to isolate the GPC gene. After S1 digestion, the DNA was repaired with the Klenow fragment of DNA polymerase, a *Bam*HI linker added and the DNA treated with *Bam*HI to produce a DNA fragment containing the entire GPC gene (LCMV nucleotides 1797 to 3366; Romanowski *et al.*, 1985). The DNA was inserted into transfer vectors (see text). To recover the N gene, plasmid Y-1-B (Matsuura *et al.*, 1986) was digested to completion with *Taq*I, the DNA fragment containing the N coding sequence was recovered, repaired with the Klenow fragment of DNA polymerase, ligated to a *Bam*HI linker, digested with *Bam*HI and DNA representing the N gene (residues 3 to 1773; Romanowski & Bishop, 1985) was inserted into transfer vectors. A schematic representation of the derivation of the LCMV N and GPC genes is given in Fig. 5.

Transfection and selection of recombinant viruses. S. frugiperda cells were transfected with mixtures of purified AcNPV DNA and recombinant transfer plasmid DNA by a modification of the procedures described by Smith *et al.* (1983). AcNPV DNA (1 μg), purified by the method of Smith & Summers (1978), was mixed with various concentrations of plasmid DNA (25 to 100 μg) and adjusted to 950 μl with HEPES-buffered saline (20 mM-HEPES, 1 mM-Na_2HPO_4, 5 mM-KCl, 140 mM-NaCl, 10 mM-glucose, pH 7.05). After precipitation with 50 μl of 2·5 M-$CaCl_2$, DNA was inoculated onto monolayers of 1×10^6 *S. frugiperda* cells in 35 mm tissue culture dishes and incubated for 1 h at room temperature. The supernatant fluids were discarded and 1·5 ml of medium containing 10% foetal bovine serum was added. After 4 days of incubation at 28 °C, the supernatant fluids were harvested and titrated in confluent monolayers of *S. frugiperda* cells. Plaques exhibiting no evidence of occlusion bodies (viral polyhedra, as determined by transmission light microscopy) were recovered and titrated on *S.*

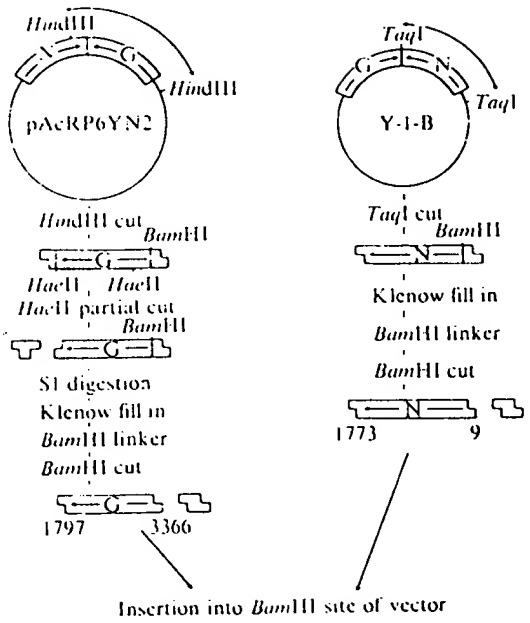


Fig. 5. Schematic diagram of the derivation of the individual LCMV S-coded N and GPC (G) genes as described in Methods. The LCMV nucleotides are numbered according to the sequence data of Romanowski *et al.* (1985). The pAcRP6YN2 and Y-1-B plasmids are those described by Matsuura *et al.* (1986).

frugiperda cells to obtain recombinant, polyhedrin-negative viruses. Following a third plaque picking, high titrated (10^7 to 10^8 p.f.u./ml) stocks of the recombinant viruses were obtained using spinner cultures of *S. frugiperda* cells.

Extraction and characterization of viral and cellular nucleic acids. Viral DNA and infected cell mRNA were prepared as described previously (Matsuura *et al.*, 1986). For Southern analyses, viral DNA preparations were digested to completion with *Bam*HI and the products resolved by electrophoresis in 0·8% agarose gel (Bethesda Research Laboratories) then blotted to Genescreen (New England Nuclear). After drying, the membranes were baked at 80 °C and hybridized to nick-translated LCMV WE DNA obtained from clone Y-1-A (Southern, 1975; Matsuura *et al.*, 1986). The membranes were then washed and autoradiographed. Cellular mRNA preparations were treated with 10 mM-methylmercuric hydroxide (Bailey & Davidson, 1976), resolved by electrophoresis in 1% gels of SeaKem agarose containing methylmercury and transferred by blotting to Genescreen. After blotting (Alwine *et al.*, 1977), the membranes were dried, baked at 80 °C for 2 h, then incubated with ^{32}P-labelled, nick translation products of the appropriate viral DNA as described by Denhardt (1966). The membranes were then washed and autoradiographed.

Protein and immunoprecipitation analyses. *S. frugiperda* cells were infected with virus at a multiplicity of 10 p.f.u./cell in 35 mm tissue culture dishes and labelled with 100 µCi of [^{35}S]cysteine (New England Nuclear; 1019 Ci/mmol) for 1 h at the indicated times using cysteine-free medium. Prior to labelling, the cells were incubated for 1 h in cysteine-free medium to reduce the intracellular pools of the precursor. In some experiments [^{35}S]methionine (Amersham, 1131 Ci/mmol) was employed using similar protocols. After the labelling periods, the media were removed, the monolayers rinsed three times with cold phosphate-buffered saline and the cells lysed either by boiling for 5 min in dissociation buffer (2·3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62·5 mM-Tris·HCl, 0·01% bromophenol blue, pH 6·8), or for immunoprecipitation analyses by extracting the cells with 500 µl of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0·15 M-NaCl, 0·05 M-Tris-HCl, 0·01 M-EDTA, 0·1% SDS, pH 7·4). Aliquots of 100 µl of the RIPA extracts were incubated with 5 to 10 µl of the appropriate antiserum for 1 h at 37 °C before addition of 25 µl of a suspension of 100 mg Protein A Sepharose CL-4B beads (Sigma) in RIPA buffer. Following a further 1 h incubation at 4 °C, the beads were recovered by centrifugation, washed three

Table 1. *Haemagglutinin activity of recombinant baculoviruses derived from transfer vectors with different lengths of the AcNPV polyhedrin leader*

Vector	5′ leader deletion*	HA activity†
pAcRP18	0	160
pAcRP6	−7	160
pAcRP22	−11	120
pAcRP20	−14	160
pAcRP21	−16	120
pAcRP3	−27	40
pAcRP5	−31	40
pAcRP7	−46	10
pAcRP1	−51	0
pAcRP8	−58	0

* Nucleotides removed from the 5′ upstream leader sequence of the AcNPV polyhedrin gene. ATG is at +1, +2, +3, see Fig. 1 and 2.

† HA activity expressed in 10^n infected *S. frugiperda* cells at 30 h post-infection (Possee, 1986).

times with cold RIPA buffer and the immune complexes that had bound to the beads were removed by boiling for 5 min in dissociation buffer, followed by centrifugation. Aliquots of the supernatant fluids or extracts of cell proteins prepared directly using dissociation buffer were subjected to electrophoresis in 10% discontinuous gels of polyacrylamide as described by Laemmli (1970). After electrophoresis the gels were stained with Kenacid Blue (Overton *et al.*, 1987) or impregnated with 2,5-diphenyloxazole (Bonner & Laskey, 1974) and exposed at −70 °C to X-ray film.

Haemagglutination assays. Cells infected with recombinant baculoviruses were assayed for HA activity as described previously (Possee, 1986).

RESULTS

The role of the 5′ polyhedrin leader sequence in haemagglutinin expression.

The levels of influenza virus HA protein synthesis reported previously using *S. frugiperda* cells infected with recombinant baculoviruses containing the influenza virus HA gene were low compared to the amount of polyhedrin protein produced in AcNPV infections (Possee, 1986). The transfer vector used to obtain HA expression (pAcRP5) lacked some 170 bp of the 5′ end of the coding region of the polyhedrin gene in addition to 31 bp of the immediate upstream sequence (Fig. 1). Matsuura *et al.* (1986) obtained higher expression of LCMV N and GPC genes using recombinants derived from transfer vector pAcRP6 (Fig. 1) than from viruses derived from pAcRP5. The pAcRP6 vector lacks only 7 bp of the 5′ upstream sequences (in addition to those of the coding region). Smith *et al.* (1985) had previously reported high levels of expression of interleukin 2 (IL-2) when using a vector (pAc373) that lacked the same nucleotides of the leader sequence as pAcRP6. In order to determine the effect of the integrity of the leader sequence on the expression of the HA gene several new transfer vectors were made with variable lengths of leader sequence (Fig. 1). The influenza HA gene was inserted into each of these vectors and recombinant viruses were derived (Possee, 1986). HA expression from the recombinant viruses was assessed at 30 h post-infection as described previously (Possee, 1986; Table 1). Increased HA expression was obtained with increased representation of the 5′ leader sequence (at least up to residue −14), although no difference in HA activity was detected between recombinants derived from vectors possessing the entire leader sequence by comparison with those missing 7, 11 or 14 bp of the leader. The kinetics of HA expression obtained with each of the recombinant viruses were similar to that reported previously for viruses derived from pAcRP5 (Possee, 1986) with a peak of activity reached by 30 to 40 h post-infection (using an m.o.i. of 10 p.f.u./cell), followed by a gradual decline from 42 h (data not shown). This contrasts with the expression kinetics reported for IL-2 which continued until 72 h post-infection (Smith *et al.*, 1985). Although the level of HA expression by pAcRP20-derived recombinants was four times greater than from viruses derived from pAcRP5, the amount of HA protein made, as assayed by radiolabelled amino acid incorporation, was still not

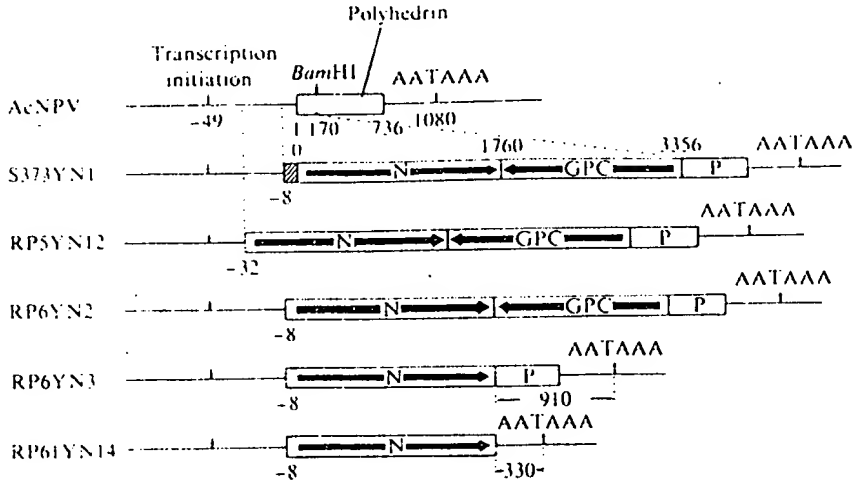


Fig. 6. Genetic organization of the recombinant viruses used to express LCMV N protein. The LCMV nucleotide positions (Romanowski *et al.*, 1985) are given above the boxed regions, the AcNPV nucleotide numbers relative to the polyhedrin gene (Fig. 1) are given below those boxes. Coding arrangements of the LCMV N and GPC genes are indicated by thick arrows, the sequences coding the AcNPV polyhedrin gene are indicated by P in the boxes. Transcription initiation and termination positions are shown by thin arrows. 3′ non-coding distances are given for two of the constructions. The hatched region of the S373YN1 recombinant represents the additional sequences present in the vector (see text and Fig. 1).

comparable to that of the polyhedrin protein made by AcNPV (data not shown). Whether the highest levels of HA observed with the different recombinant viruses reflected the optimal expression of the system for this protein, or the particular characteristics of the assay for cell-associated HA has not been determined.

Preparation of recombinant baculoviruses containing the LCMV N and GPC genes

Previous studies described the construction of recombinant baculoviruses containing the ambisense LCMV S DNA orientated so that it would express either the viral N or GPC proteins (Matsuura *et al.*, 1986). Three transfer vectors were employed to produce the recombinant baculoviruses, namely pAcRP1, pAcRP5 and pAcRP6 (see Fig. 1, 2). Although more LCMV protein was synthesized in cells infected with recombinant viruses derived from the pAcRP6 vector than from those made from the pAcRP5 vector, little if any N or GPC protein was detected for recombinants derived from pAcRP1. In addition, protein analyses indicated that the level of expression of the LCMV N and GPC proteins synthesized by pAcRP5- and pAcRP6-derived recombinants was not as high as the level of polyhedrin protein synthesized by AcNPV. A variety of reasons could account for this observation, reasons relating to the character of the polyhedrin promoter, the particular constructions represented in the recombinant viruses, or certain intrinsic characteristics of the inserted genes or their products.

A study was instigated to determine the reason for the lower levels of LCMV gene expression. Initially the question of whether the lower expression was specific to pAcRP-derived recombinants was investigated. As mentioned above, comparison with published data indicated that the pAcRP6 vector used to produce the N and GPC recombinants lacked the same 5′ upstream nucleotides as the pAc373 vector originally described by Smith *et al.* (1983). The LCMV S DNA was therefore inserted into the pAc373 vector kindly provided by Dr Max Summers (Texas A & M University, College Station, Tex., U.S.A.) and a recombinant virus produced, S373YN1, with the LCMV N gene in the 5′ orientation (Fig. 6). However, when the 5′ insertion sequence of the pAc373-derived recombinant transfer vector was analysed it was found that it contained some non-viral sequences that presumably came from contaminants in

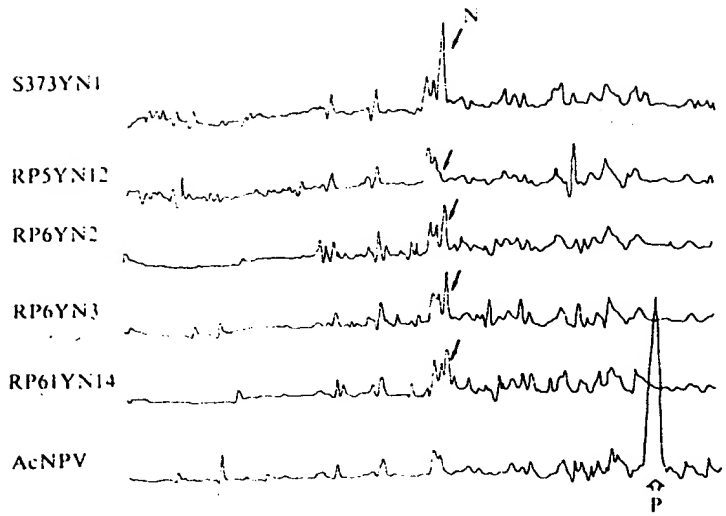


Fig. 7. Traces of the stained proteins resolved by gel electrophoresis of extracts of *S. frugiperda* cells infected with recombinant viruses expressing LCMV N protein (arrow) or AcNPV. The AcNPV polyhedrin protein is indicated (P). After polyacrylamide gel electrophoresis of infected cell extracts, the cells were stained with Kenacid Blue (Overton *et al.*, 1987) and scanned at 410 nm using a densitometer.

the linker originally employed to make pAc373 (see additional underlined sequence in Fig. 1 and the hatched area in Fig. 6). The recombinant virus S373YN1 and the previously described RP5YN12 and RP6YN2 viruses were used at similar multiplicities to infect *S. frugiperda* cells. Extracts of the infected cell proteins were resolved by gel electrophoresis and the gel was scanned optically to determine the relative amounts of LCMV N protein present. By comparison with the polyhedrin protein made in AcNPV-infected cells there was substantially less N protein made by the recombinant S373YN1, although the latter made more N protein than RP6YN2, which in turn made more than RP5YN12 (Fig. 7). The difference in level of expression between pAcRP6- and pAc373-derived recombinants was reproduced with recombinants independently derived from the two vectors and in other analyses of the proteins synthesized by the recombinants. However, although it was clear that the pAc373-derived virus did not make N protein at levels comparable with that of the AcNPV polyhedrin gene, it was not possible to determine from these data whether the difference in the levels of expression between S373YN1 and RP6YN2 was due to the different origins of the vectors (and thence the recombinant viruses), or to the presence of the extra sequences in the 5′ upstream region of the pAc373 transfer vector (Fig. 1).

LCMV S DNA has an ambisense arrangement with the N gene coded in one half of the DNA and GPC on the other strand of the other half of the DNA. In addition it has a unique intergenic region (Romanowski *et al.*, 1985). In view of these properties the possibility that this coding arrangement might inhibit efficient expression was examined. Recombinants were constructed with only the LCMV N gene as described in the Methods section and illustrated in Fig. 6 for the recombinant RP6YN3. When this virus was used to synthesize LCMV N protein in *S. frugiperda* cells it made no more N protein than the RP6YN2 recombinant (Fig. 7). It appeared therefore that the presence of the LCMV intergenic and GPC sequences in the latter recombinant were not inhibitory.

The role of the residual 3′ polyhedrin coding sequences in gene expression

In case the residual polyhedrin coding sequences in the recombinant viruses were deleterious to the expression of a foreign gene, the LCMV N gene was next inserted into the pAcRP61 transfer vector, a vector that lacked all the coding sequences of the polyhedrin gene as well as 13

3′ nucleotides (Methods; Fig. 3). The recombinant virus RP61YN14 was derived (Fig. 6). When this virus was used to infect *S. frugiperda* cells it also made LCMV N protein in amounts equivalent to those made by recombinants RP6YN2 and RP6YN3 (Fig. 7).

Southern analyses of the viral DNA of the various recombinants described above confirmed the genotypes of the above viruses with respect to the LCMV sequences (Fig. 8*a*). Northern analyses of the S373YN1, RP5YN12 and RP6YN2 virus-induced mRNA species indicated that, as described previously for RP5YN12, the LCMV-specific mRNA made in infected cells exhibited a broad size range, whereas mRNA species recovered from RP6YN3- and RP61YN14-infected cells (i.e. those lacking the LCMV GPC coding sequences) were more homogeneous (Fig. 8*b*). In view of the fact that the size ranges of the mRNA species from all five recombinants overlapped, the data suggested that there was premature termination of mRNA synthesis for the S373YN1, RP5YN12 and RP6YN2 recombinants, perhaps due to the LCMV intergenic or other sequences.

In summary, these studies indicated that removal of the polyhedrin coding sequences and unnecessary LCMV DNA sequences did not change the level of expression of LCMV N protein. Although viruses derived from the pAc373 vector expressed higher levels of LCMV N protein than viruses obtained from pAcRP6, the amount made was still not equivalent to the expression of the AcNPV polyhedrin protein.

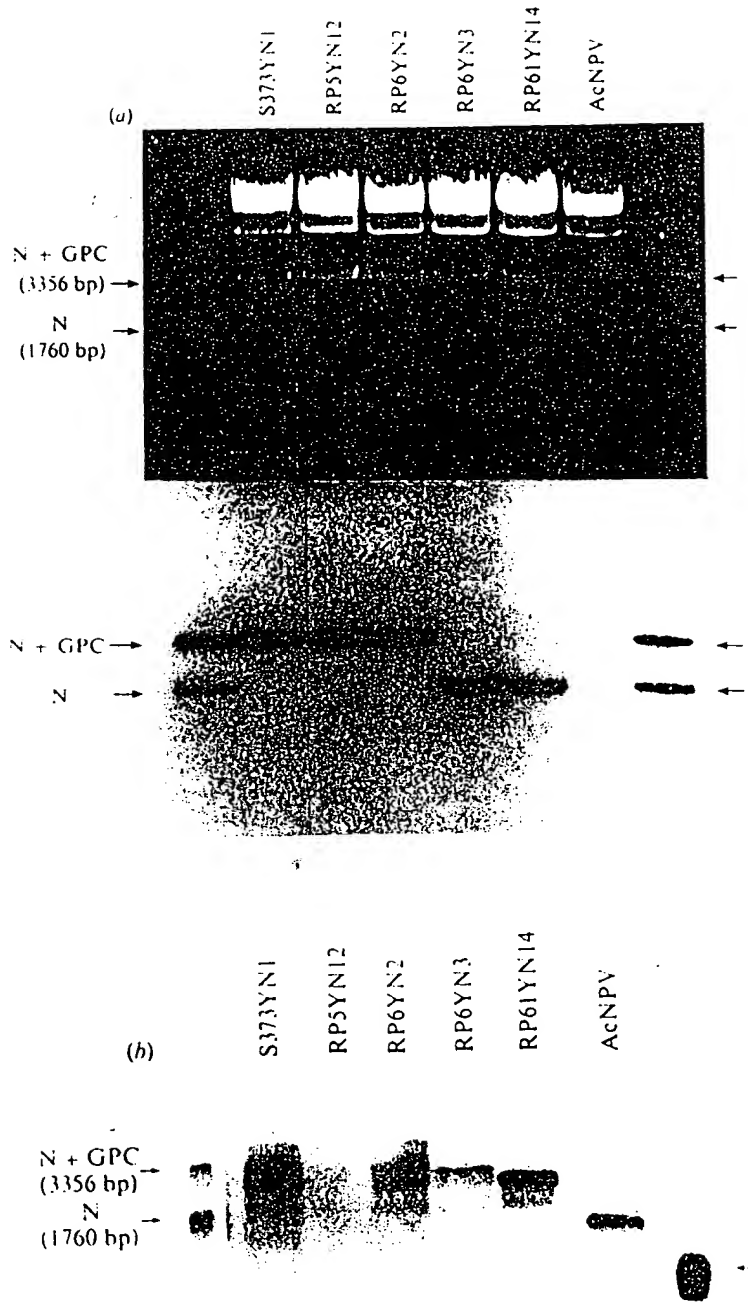
High level expression of LCMV N protein by recombinants possessing the complete upstream sequence of the polyhedrin gene

It had been observed that the amount of N protein made by the pAcRP1-, pAcRP5- and pAcRP6-derived transfer vectors increased with the increasing representation of the immediate 5′ upstream sequences of the polyhedrin gene (Matsuura *et al.*, 1986). This observation raised the possibility that the reason for the low level of expression of LCMV N protein by the recombinant viruses was that some of the 5′ upstream sequences were absent from the pAcRP1-, pAcRP5- and pAcRP6-based recombinants. Another transfer vector was therefore made, pAcYM1, that contained all of the polyhedrin upstream sequences, plus the A of the initiating ATG, but lacking all of the other polyhedrin coding sequences and 13 of the 3′ downstream nucleotides (Methods; Fig. 1, 2, 4). The LCMV N gene was inserted into this vector and the recombinant virus YM1YN1 was recovered. By comparison to the previously described recombinants RP6YN3 and RP61YN14 (see Fig. 2, 6) the amount of labelled LCMV N protein made by the YM1YN1 recombinant was considerably greater and at least equivalent to the amount of polyhedrin protein made by AcNPV-infected cells (Fig. 9).

Since the YM1YN1 recombinant lacked the entire coding region of the polyhedrin gene, other than the A of the initiating ATG, the question of the effect of the presence of the carboxy-terminal two-thirds of the coding region of the polyhedrin gene and the 13 3′ downstream nucleotides (Fig. 2, residues 2358 to 2937) on the expression of LCMV N protein was investigated. A recombinant transfer vector was constructed from *Mst*II-*Xho*I restriction fragments recovered from the vectors pAcYM1YN1 and pAcRP6YN3 so that the derived plasmid, pAcYM1YN1-3′, now contained two-thirds of the coding region of the polyhedrin gene and all the downstream sequences, as exemplified in schematic form in Fig. 10. When the resulting recombinant virus, YM1YN1-3′, was used to express the LCMV N protein, again high levels of N protein were made (Fig. 9) as determined from both fluorograms and stained patterns of the infected cell proteins. This result confirmed the previous conclusion that the presence of the residual polyhedrin coding sequences in recombinant viruses was not deleterious to foreign gene expression.

Identification of an upstream deletion in the pAcRP6 transfer vector

To determine whether there were differences between the pAcYM1, pAcRP6 and pAc373 transfer vectors other than the immediate 5′ upstream sequences of the polyhedrin coding region, *Hin*fI analyses of the plasmids were undertaken. As shown in Fig. 11, the small *Bam*HI-*Xho*I restriction fragment derived from all three plasmids exhibited a difference in the *Hin*fI profile for pAcRP6. Apart from the indicated change (and differences that could be accounted for by


(a)
S373YN1
RP5YN12
RP6YN2
RP6YN3
RP61YN14
AcNPV
N + GPC
(3356 bp)
N
(1760 bp)
N + GPC
N
(b)
S373YN1
RP5YN12
RP6YN2
RP6YN3
RP61YN14
AcNPV
N + GPC
(3356 bp)
N
(1760 bp)
Polyhedrin
(726 bp)

the orientation of the inserted sequences in pUC8), no other difference was detected in the *Hin*fI patterns of either the large or small *Bam*HI-*Xho*I fragments. Sequence analyses on the strand-separated products showed that fragments *b* (pAcYM1) and *d* (pAc373) were identical (data not given). Likewise fragments *a* (pAcYM1), *c* (pAc373) and one set of strand-separated products from *c* (pAcRP6) were also identical (data not given). However band *c* of pAcRP6 was found to contain a second restriction fragment that by sequence analyses of its strand-separated products was found to be similar to *b* and *d* but shorter due to an internal deletion of some 60 bp. Results similar to those of pAcRP6 were obtained for pAcRP61. Sequence analyses (unpublished data) localized the deletion to some 2000 nucleotides upstream of the polyhedrin coding region of AcNPV. On the basis of the flanking sequences, it has been determined that the deletion is in an open reading frame, although it is not known whether it is transcribed and translated, or whether it corresponds to an essential or non-essential gene product of the virus. If the gene was essential then virus genomes containing the deletion would not be viable. Alternatively, if the gene was not essential then recombinant genomes may be viable but exhibit an altered phenotype. If the change included reduced expression of a downstream gene then this could be a reason for the difference in expression between pAcRP6- and pAc373-derived recombinant viruses.

In order to determine whether the deleted sequences of pAcRP61 play a role in the lower expression of its derived recombinants, the *Mst*II-*Eco*RV restriction fragments of pAcRP61YN14 (containing the upstream deletion, see triangles in Fig. 10) and equivalent pAcYM1YN1 plasmid (lacking the deletion) were exchanged as exemplified in the centre and left hand panels of Fig. 10. Two recombinant transfer vectors were derived, namely pAcYM1YN1-d and pAcRP61YN14-r. They were used to produce the recombinant viruses YM1YN1-d and RP61YN14-r, respectively. When these recombinants were employed to infect *S. frugiperda* cells, the recombinant RP61YN14-r (derived from the vector that did not have the deletion) synthesized a low quantity of N protein, equivalent to that made by RP61N14. By contrast, the YM1YN1-d recombinant (derived from the vector containing the deletion) synthesized a large amount of N protein, essentially equivalent to that made by YM1YN1 (Fig. 9). *Hin*fI restriction enzyme analyses of the smaller *Xho*I-*Eco*RV fragments obtained from *Eco*RI subclones of RP61YN14 and YM1YN1-d demonstrated that the viruses did not in fact contain the upstream deletion (data not shown). Whether all viable recombinants derived from the RP6 transfer vectors lack the deletion is not known. Analyses of other, independently produced recombinant viruses are required to investigate that question. However, the results obtained for the RP61YN14 and RP61YN14-r recombinants indicate that the upstream deletion in the pAcRP6 transfer vector is not responsible for the difference in expression between the pAc373- and pAcRP6-derived recombinant viruses. Unless there are other sequence changes that have

Fig. 8. Southern and Northern blot analyses of recombinant baculovirus nucleic acids. In (*a*) *Bam*HI digests of viral DNA were recovered from AcNPV, the recombinant viruses RP5YN12, RP6YN2 and S373YN1 derived (respectively) from the pAcRP5, pAcRP6 or pAc373 transfer vectors containing the complete LCMV S DNA (Matsuura *et al.*, 1986; Smith *et al.*, 1983), a recombinant RP6YN3 obtained from a pAcRP6 transfer vector containing only the N gene of LCMV, and a recombinant RP61YN14 obtained from a pAcRP61 transfer vector containing the LCMV N gene but lacking the residual 3′ coding region of the AcNPV polyhedrin gene (see Fig. 6). The DNA products were resolved by agarose gel electrophoresis (top) and probed with nick-translated LCMV WE S DNA (bottom). The probe was derived from a 3·4 kb *Bam*HI restriction fragment recovered from clone Y-1-A (Matsuura *et al.*, 1986). Controls for the Southern analyses included the *Bam*HI fragments representing the 3356 bp LCMV N and GPC sequences and the 1760 bp LCMV N gene sequences (arrows). In (*b*) total cellular RNA was extracted from *S. frugiperda* cells infected with AcNPV, or the recombinant viruses used for the Southern analyses, the poly(A)$^+$ RNA species were recovered and treated with methylmercuric hydroxide and then resolved by electrophoresis in a 1% agarose gel containing 10 mM-methylmercuric hydroxide. After blotting and fixing to Genescreen, the RNA was hybridized to nick-translated probes. For the recombinant viruses an *Mst*II 1·1 kb DNA was used as a probe for the N gene sequences (Romanowski & Bishop, 1985). For the AcNPV lane a 726 bp fragment representing the polyhedrin coding region was employed. Size markers (treated similarly) consisted of the 726 bp polyhedrin sequence (right side), and a mixture of *Bam*HI fragments representing the 3·4 kb LCMV S DNA and the 1·76 kb N gene (left side).

Polyhedrin (726 bp)

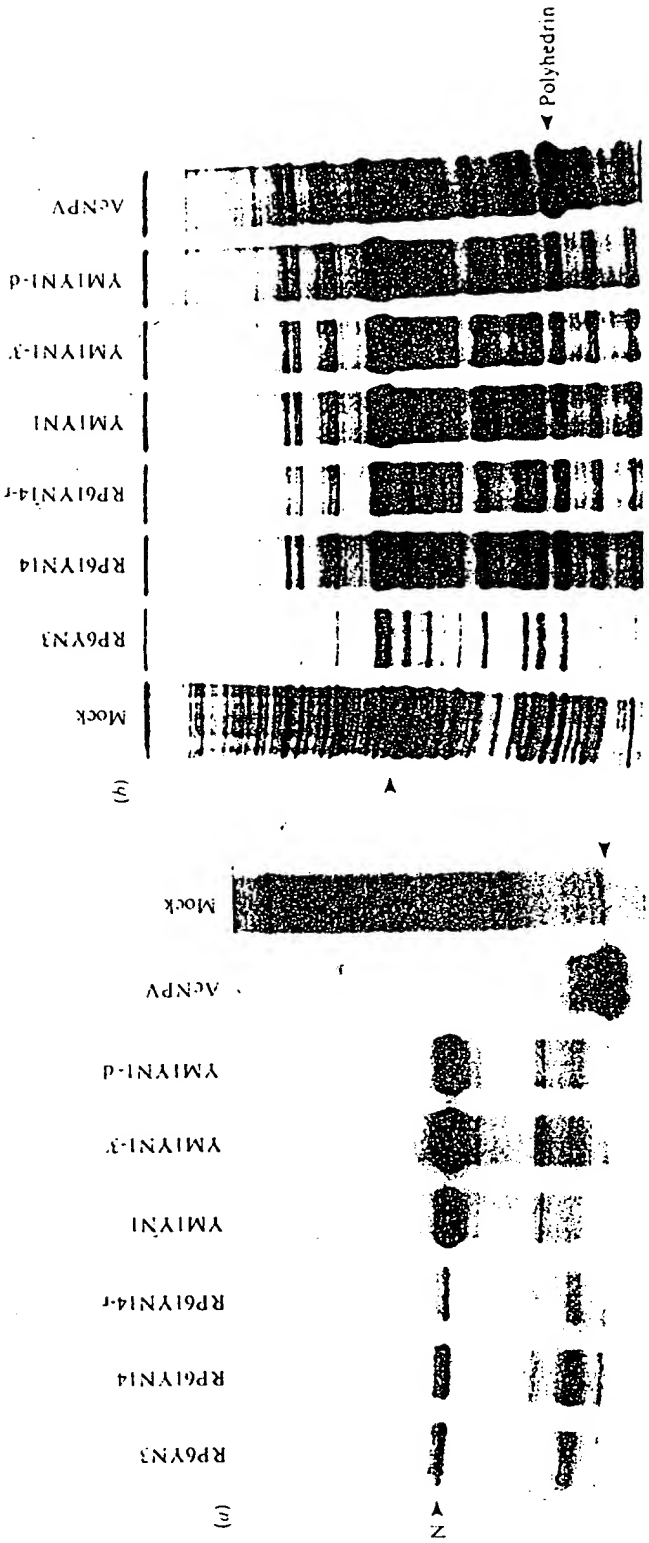


Fig. 9. Expression of LCM proteins by recombinant baculoviruses. *S. frugiperda* cells were infected by recombinant viruses containing only the N gene with (RP6YN3, YM1YN1-3'), or without (RP61YN14, YM1YN1-d, RP61YN14-r), the residual 3' coding region of the polyhedrin gene (see Fig. 6.10). Cells were also infected with wild-type AcNPV or were mock-infected. Proteins were labelled at 24 h post-infection for 1 h with [^{35}S]methionine, the labelled proteins recovered and resolved by gel electrophoresis. Gels were either stained and photographed (*a*) or fluorographed (*b*). The positions of the LCMV N and AcNPV polyhedrin proteins are indicated.

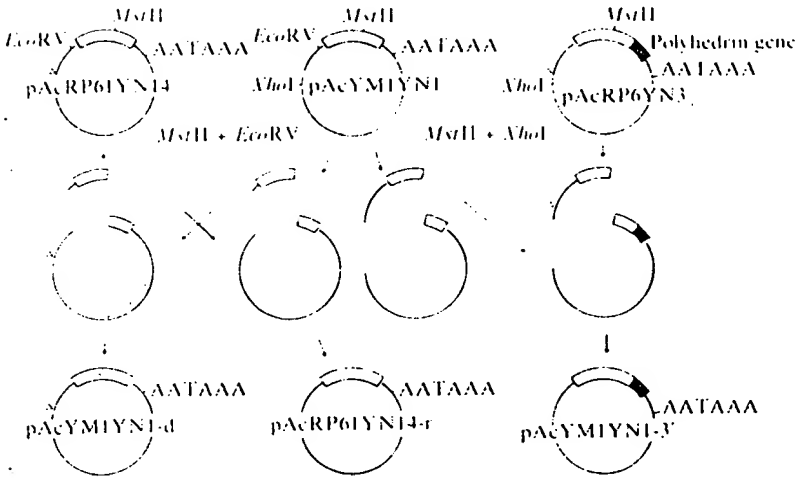


Fig. 10. Schematic diagrams of the construction of transfer vectors pAcYM1YN1-d, pAcRP6IYN14-r and pAcYM1YN1-3′ as described in the text.

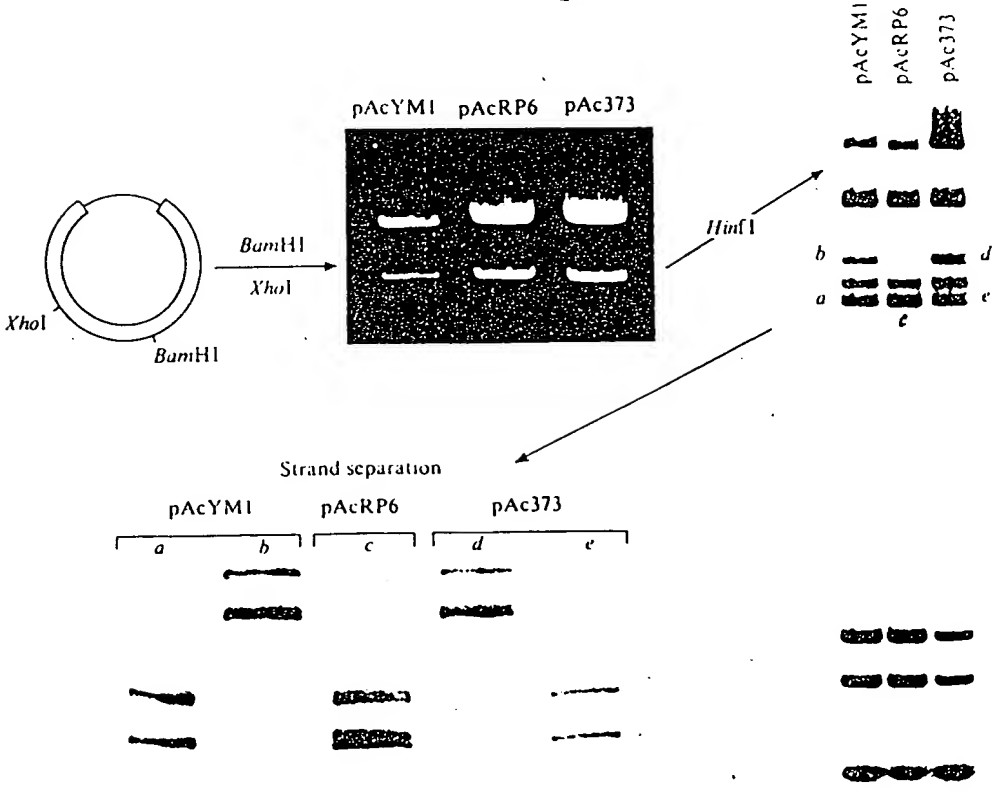


Fig. 11. Analyses of the upstream sequences of pAcYM1, pAcRP6 and pAc373 transfer vectors. The small *Bam*HI *Xho*I restriction enzyme fragments derived from the three transfer vectors were recovered, digested with *Hin*fI (right panel) and fragments *a*–*e* recovered. Each fragment preparation was strand-separated with the results shown in the lower left panel.

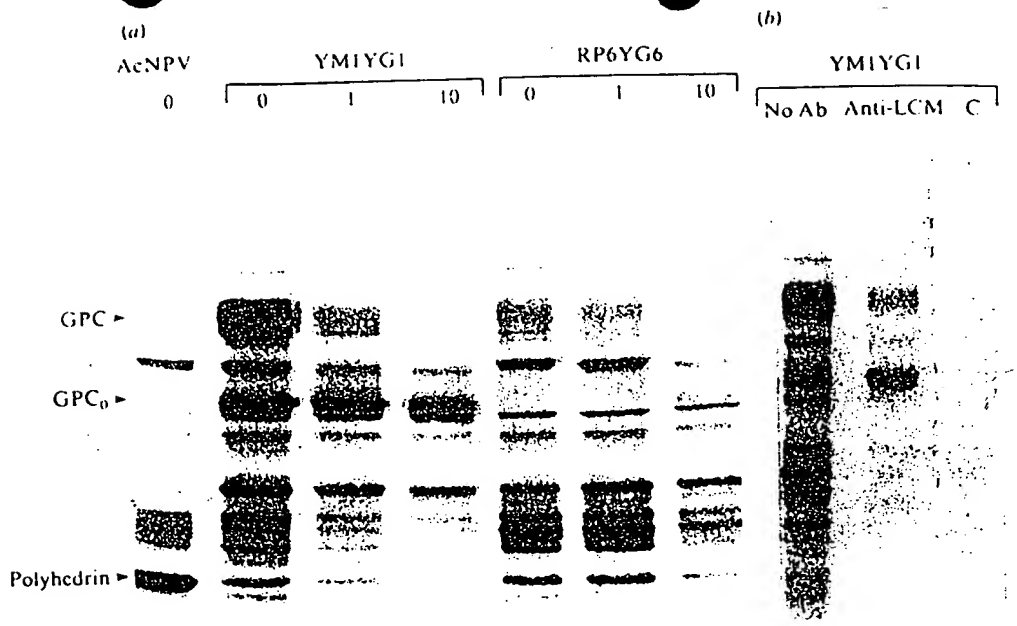


Fig. 12. (*a*) Effect of tunicamycin (Tm) on the expression of GPC by recombinant baculoviruses derived from pAcRP6 and pAcYM1 transfer vectors. Confluent monolayers of *S. frugiperda* cells were infected at multiplicities of 10 p.f.u./cell with wild-type AcNPV, or the recombinant viruses YM1YG1 or RP6YG6 (derived from pAcYM1 and pAcRP6, respectively; see Matsuura *et al.*, 1986) and incubated at 28 °C. For the Tm-treated cells, the media were replaced 18 h post-infection with medium containing Tm at either 0, 1 or 10 μg/ml as indicated. After an additional 6 h of incubation at 28 °C, the cells were starved in methionine-free media containing the appropriate amounts of drug, then labelled for 1 h in the same media containing 15 μCi [^{35}S]methionine per ml. The cell monolayers were rinsed, lysed in RIPA buffer and extracts resolved by electrophoresis in a 10% polyacrylamide gel as described by Laemmli (1970), followed by fluorography. The positions are indicated for the AcNPV polyhedrin protein, the LCMV GPC and expected 57×10^3 mol. wt. non-glycosylated precursor, GPC_0 (Romanowski *et al.*, 1985). (*b*) Immunoprecipitation of the approx. 72×10^3 mol. wt. GPC and putative GPC_0 proteins. Samples of the YM1G1-infected cells were immunoprecipitated with a polyclonal antiserum to LCMV or with non-immune control (*c*) antiserum as described in Methods.

not been identified, it is likely that the difference in the level of expression of the LCMV N protein is primarily accounted for by the difference in representation of the immediate upstream sequences of the polyhedrin gene for recombinants originating from pAcRP6 and pAcYM1.

High level expression of LCMV GPC protein by recombinants possessing the complete upstream sequence of the polyhedrin gene

The LCMV GPC gene was inserted into the pAcYM1 vector, and the transfer vector pAcYM1YG1 was recovered. Using this vector the recombinant virus YM1YG1 was obtained. By comparison with recombinant RP6YG6 (derived from the pAcRP6 transfer vector; Matsuura *et al.*, 1986), the amount of labelled LCMV GPC protein made in infected *S. frugiperda* cells by the YM1YG1 recombinant was considerably greater than that obtained with RP6YG6. At least two other bands of protein that migrated ahead of the GPC protein appeared to be virus-induced. These proteins were not present in AcNPV-infected cells (Fig. 12), nor in uninfected cells. The smaller of these species migrated with a mobility expected for the unglycosylated form of GPC (GPC_0; Romanowski *et al.*, 1985). Both appeared from immunoprecipitation studies to be related to the LCMV glycoprotein (Fig. 12). Peptide analyses are required to confirm the relationships of these expressed proteins or to determine whether they represent degradation or premature termination (or other) products of the LCMV gene.

The amount of GPC protein made in the recombinant virus-infected cells was estimated to be of the order of 20% of the labelled cell proteins (Fig. 12). Analyses of stained gels infected with the YM1YG1 recombinant also indicated that some 20% of the cell protein was the LCMV glycoprotein (data not shown). As noted previously (Matsuura *et al.*, 1986), the GPC protein migrated as a broad band in polyacrylamide gels, presumably due to the presence of various glycosylated forms. In order to obtain evidence that the GPC protein was glycosylated and to determine if either of the other faster moving bands was also glycosylated, the effect of the drug tunicamycin was investigated. At concentrations of 1 and 10 μg per ml of culture fluids, tunicamycin inhibited the synthesis of GPC and the minor LCMV-induced protein that migrated between the GPC and putative GPC_n protein (Fig. 12). Although, particularly at the higher concentration of tunicamycin, there were reduced quantities of all the labelled proteins, the putative GPC_n was not inhibited to the extent of GPC. However there was no increase in any band concomitant with the decrease in GPC. These observations suggest that the largest two proteins were *N*-glycosylated, although the incorporation of appropriate labelled carbohydrate precursors will be needed to confirm this hypothesis. It had been expected that there would be a corresponding increase in the amount of unglycosylated GPC (GPC_0) with the inhibition of glycosylation. Why this was not observed is not known.

Electron micrographs of S. frugiperda *cells infected with recombinant baculoviruses*

In view of the amounts of LCMV N and GPC proteins made by the YM1-derived recombinant viruses, an examination of electron micrographs of cells infected with the YM1-derived recombinant viruses was undertaken (Fig. 13). The micrographs were compared with uninfected cells (Fig. 13*a*), cells infected with AcNPV (Fig. 13*b*) and cells infected with a recombinant virus derived from pAcRP1 containing LCMV S DNA that previous analyses showed did not make detectable amounts of LCMV N protein (Fig. 13*d*; Matsuura *et al.*, 1986). For the YM1YN1 recombinant virus that expressed large quantities of LCMV N protein, many inclusion bodies were evident in the cytoplasm of infected cells (Fig. 13*c*). For the YM1YG1-infected cells, although no inclusion bodies were evident, the cytoplasm of the cells was seen to be highly vacuolated (Fig. 13 *e, f*), and there was a dense endoplasmic reticulum surrounding the nucleus. As expected, non-occluded viruses were evident in the nuclei of all the recombinant virus-infected cells.

DISCUSSION

Data have been obtained with recombinant baculoviruses which indicate that the level of expression of a foreign protein that replaces the AcNPV polyhedrin gene is related to the representation of the 5′ upstream sequence of that gene. At least for the expression of the LCMV N protein, none of the coding sequences of the baculovirus polyhedrin gene, or 13 of the immediate downstream nucleotides, appears to be required for high level expression of a foreign protein. By contrast, the results suggest that the immediate 5′ upstream sequences are important for high level expression.

In the studies described in this paper a variety of transfer vectors have been produced and used to make recombinant viruses that express the influenza virus HA protein. Using a cell-associated influenza virus HA assay, higher expression was obtained with increasing representation of the polyhedrin upstream sequences. However this correlation was apparently only valid up to the −14 deletion (Table 1). Recombinants with smaller deletions (0, −7, −11) did not appear to make more HA, although whether this was because extra HA protein was made and exported from infected cells, or whether the observation only reflected a limitation of the cell-associated HA assay, has not been investigated. Protein analyses of infected cells and their supernatant fluids are required to resolve that point.

For recombinants containing the LCMV genes, higher protein expression has been demonstrated for viruses derived from a new transfer vector pAcYM1 (which has all the upstream sequences of the AcNPV polyhedrin gene including the A of the initiating ATG codon), than for recombinant viruses recovered from a transfer vector, pAcRP6, which lacks the first seven nucleotides of the upstream sequence as well as the polyhedrin ATG. In fact, the level

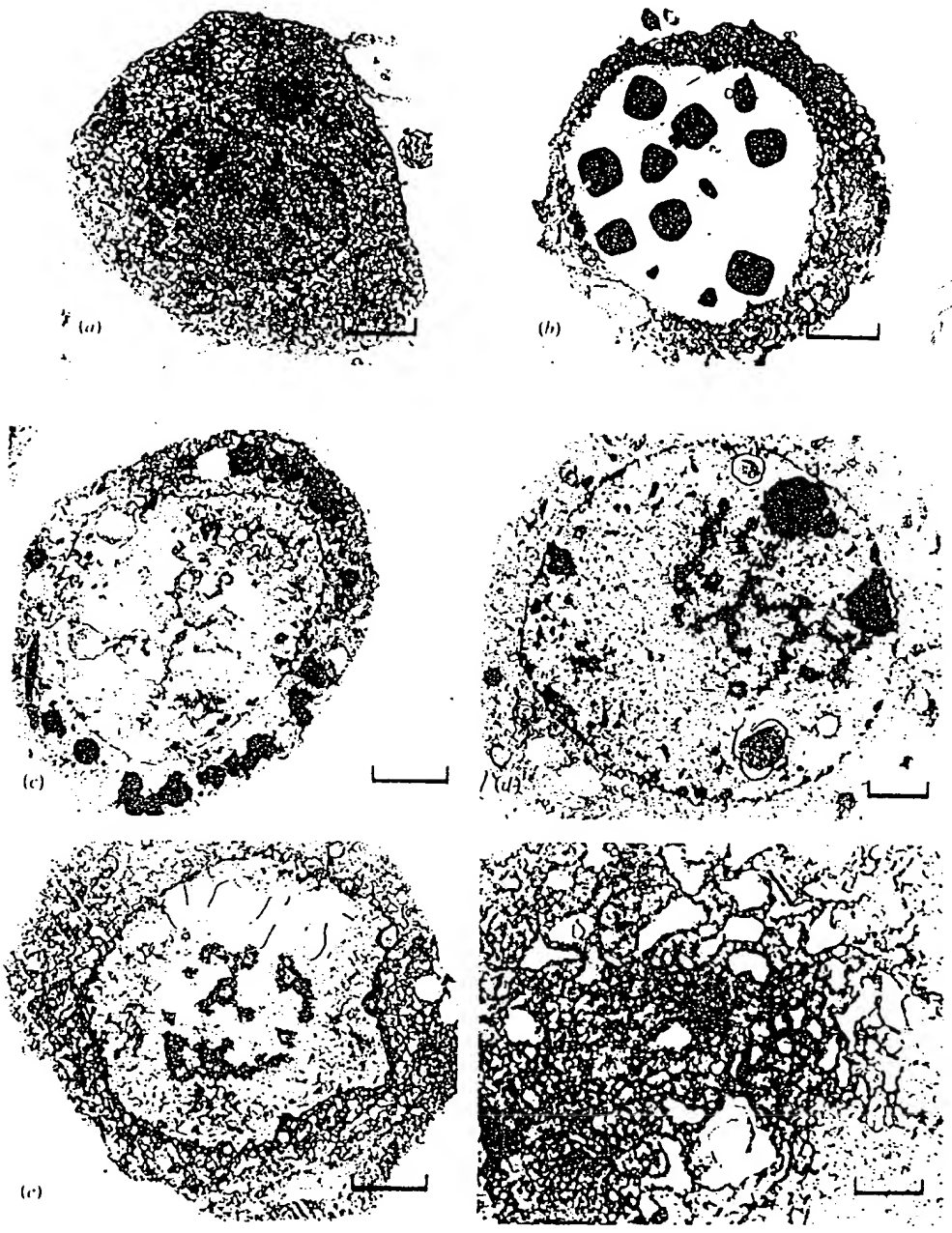


Fig. 13. Electron micrographs of uninfected or infected *S. frugiperda* cells. The panels show (*a*) uninfected *S. frugiperda*, (*b*) AcNPV-infected *S. frugiperda*, (*c*) *S. frugiperda* infected with recombinant YM1YN1, (*d*) *S. frugiperda* infected with recombinant A251 derived from pAcRP1 (Matsuura *et al.*, 1986), (*e*) *S. frugiperda* infected with recombinant YM1YG1 and (*f*) cytoplasm of *S. frugiperda* infected with recombinant YM1YG1. Bar markers represent for (*a*), (*b*), (*c*) and (*e*) 5 μm, (*d*) 2 μm and (*f*) 1 μm.

of expression of the LCMV N gene by the recombinant YM1YN1 derived from pAcYM1 rivalled that of polyhedrin protein expressed by AcNPV (Fig. 9). In this connection it is perhaps noteworthy that the flanking sequences of the initiating ATG translation codons of the polyhedrin gene (Fig. 2) and the LCMV N and GPC genes (Romanowski & Bishop, 1985;

Romanowski *et al.*, 1985) are similar (AATATGC, AAAATGT and AGGATGG, respectively), although whether these have any bearing on the level of translation of the respective gene products in *S. frugiperda* cells is not known. Infection by YM1YN1 resulted in numerous inclusion bodies in the cytoplasm of infected *S. frugiperda* cells (Fig. 13). For another recombinant virus derived from pAcYM1 that expresses the LCMV GPC protein (YM1YG1), the level of GPC protein synthesis was also significantly higher than for recombinant RP6YG6, derived from the pAcRP6 vector (Fig. 12). *S. frugiperda* cells infected with YM1YG1 exhibited an extensive cytoplasmic vacuolation (Fig. 13). The reason for the vacuolation is not known. It may be a result of the presence of large quantities of glycoprotein in the endoplasmic reticulum, Golgi apparatus and/or plasma membrane of the infected cells. How a cell responds to the synthesis of large amounts of glycoprotein is an area for future investigation.

Although the pAcRP6 transfer vector and its derivatives were found to have an upstream deletion in an open reading frame, the presence of the deletion in the vector did not account for the reduced expression of a foreign gene by derived recombinants. In fact, analyses of two recombinants obtained from vectors which had the deletion showed that the deletion was not present in the recombinant viruses, presumably due to the sites of recombination between the AcNPV DNA and the DNA of the transfer vectors. Whether all recombinants derived from pAcRP6 lack the deletion because it is in an essential gene product is not known.

The fact that recombinants derived from pAc373 reproducibly made somewhat more LCMV N protein than recombinants derived from pAcRP6 (Fig. 7) may be of interest. Unless there are other reasons that account for the difference, the only known sequence difference between the two transfer vectors is the presence of some extra non-viral sequences in the pAc373 transfer vector. These sequences resemble a *Bam*HI linker and may have originated from impurities present in the linker used to prepare the vector. It is possible that they act as a partial substitute for the missing polyhedrin leader sequences allowing higher levels of expression. Whether the higher levels of expression by the pAc373- or YM1YN1-derived recombinants are due to higher rates of transcription or translation or even stability of the mRNA species is not known.

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